University of Alberta

Mechanisms of matrix metalloproteinase-induced vascular dysfunction in endotoxemia

by

Jonathan James Cena

©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmacology

Edmonton, Alberta
Fall 2008

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-46293-5
Our file Notre référence
ISBN: 978-0-494-46293-5

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Septic shock is the leading cause of death in intensive care units in Canada and the USA. Lipopolysaccharide (LPS) initiates the development of gram negative sepsis by stimulating the expression of inflammatory mediators which result in vascular hyporeactivity to vasoconstrictors, defined as an insensitivity of the vasculature to vasopressor agents. Nitric oxide (NO) is a key mediator contributing to this vascular dysfunction associated with septic shock and can combine with superoxide, to form peroxynitrite, a toxic oxidant. Peroxynitrite can activate matrix metalloproteinases (MMPs) which are involved in various cardiovascular diseases. However, their role in septic shock is unknown.

Using the isolated thoracic rat aorta, I studied three experimental models which mimic features of septic shock to explore the role of MMPs in the development of vascular dysfunction in endotoxic shock. I discovered that: MMP inhibition protected against LPS-induced vascular hyporeactivity *in vitro*; *in vivo* LPS increased MMP activity in aortae of LPS-injected rats; and, MMP inhibition protected against interleukin-1β-induced vascular hyporeactivity *in vitro*.

The endothelium regulates both vascular function and the immune response; therefore, its role in the development of vascular hyporeactivity concerning MMPs was assessed. MMP inhibition prevented LPS-induced vascular hyporeactivity in endothelium-intact but not denuded vessels. MMP activity was enhanced in endothelium-intact but not denuded vessels after LPS stimulation. These results implicate the endothelium in MMP-induced vascular hyporeactivity induced by LPS.

In order to examine the effect of MMP inhibition *in vivo* we administered LPS and the MMP inhibitor, doxycycline, into rats. After six hours aortae were isolated and the vasoconstrictor response to phenylephrine was tested *in vitro*. Doxycycline protected against LPS-induced development of vascular hyporeactivity.

In order to test the mechanisms of interleukin-1β-induced vascular hyporeactivity *in vitro,* I examined the contribution of the NO/cGMP pathway with respect to MMPs. Inhibition of NO synthase or soluble guanylate cyclase protected against interleukin-1β-induced vascular hyporeactivity and prevented the increase in MMP activity *in vitro.*

Results from this thesis will contribute to a better understanding of the mechanisms of vascular hyporeactivity to vasoconstrictors in sepsis-like conditions, and introduce a potential new therapeutic target for the treatment of this disease.

Dedicated to my altruistic Mother, for her endless support and love

Acknowledgements

Dr. Richard Schulz:
Thank you for giving me the opportunity to work in your laboratory. It has been an incredible journey together and I've learned a lot from you, not just as a supervisor but as a friend. You have taught me that science isn't just about testing a hypothesis, but it's also about presentation and communication. Furthermore, you have also taught me to approach science with an open mind, and to believe in my results; the results never lie. I want you to always remember that your wise advice has made me a both a better scientist and a better person.

Ava Chow:
There hasn't been a day where you haven't done something novel or thought of something brilliant. There also hasn't been a day that I could have gone through without your advice and your friendship. You never cease to amaze me. When you're saving the world and winning Nobel prizes, don't forget about all of us, because we will never forget you.

Dr. Manoj Lalu:
Thank you for taking time away from your superhuman schedule to teach me literally all of the techniques I needed to utilize for my thesis work. We've worked together endless hours and developed a great friendship at the same time.

Technicians:
I have been blessed to work with extremely talented technicians during my time in the Schulz lab. Thank you *Jolanta Sawicka* for all of your assistance during my first few years of graduate school. I will never understand how you were capable of keeping the laboratory in line. You've always kept everything (and everyone) under control while still doing great science. Thank you *Cory Rosenfelt* for your friendship and support. I hope you succeed in your professional careers. Thank you *Kristy Martens* for your friendship and support during my final months in the laboratory. We are truly grateful to have you both as a technician and a friend.

Past and present students in the Schulz lab:
Thank you *Dr. Ahmed El-Yazbi* for all of your expert advice. You always knew everything about any subject. I am excited to see your great contributions to science in the future. Thank you *Dr. Hernando Leon* and *Mohammad Ali* for your friendship and support. We have spent endless hours (past midnight to early morning) in the laboratory together. My training wouldn't have been as great without you both; you will not be forgotten.

Summer students:
Thank you *Shakib Rahman, Christine Reimer, Mariel Bagdan, Ashima Sharma, Saira Butt, Jan Schulz, Tyler van Mulligan*, and *Anna Lam* for being exceptional summer students. I have learned a great deal from each and every one of you. Working with all of you has taught me to be a better teacher.

Post-Doctoral fellows and research associates:
Thank you *Dr. Greg Sawicki, Dr. Meltem Sariahmetoglu, Dr. Serena Viappiani, Dr. Bryan Crawford, Dr. Adrian Nicolescu*, and *Dr. Arul Kandesamy*. I admire your dedication to science.

Secretaries:
Thank you *Judy Deuel, Joy Pedersen, Sharon Kilback, Francis Day*, and *Mary-Jo Boeglin* for providing administrative support throughout my degree.

Supervisory Committee:
Thank you *Dr. Sandy Clanachan* and *Dr. Susan Jacobs* for your support over the past 4 years. You have taught me to keep an open mind during my scientific investigations, as well as approach problems from different angles. These skills will help me in my future endeavours and I thank you for that.

Table of Contents

CHAPTER 1
INTRODUCTION

1.1 Pathophysiology of septic shock 2

1.1.1 Definition of septic shock 2
1.1.2 Pathogenesis of septic shock 4
1.1.2.1 Lipopolysaccharide 4
1.1.2.2 Pro-inflammatory cytokines 6
1.1.3 Vascular manifestations of septic shock 8
1.1.3.1 Vascular hyporeactivity 8
1.1.3.2 Role of the endothelium in septic shock 9
1.1.3.3 Models of vascular hyporeactivity 10
1.1.4 Molecular mechanisms of pathogeneity 13
1.1.4.1 Nitric oxide 13
1.1.4.2 Oxidative stress 16

1.2 Matrix metalloproteinases 19

1.2.1 Classification and structure 19
1.2.2 Regulation and function 21
1.2.3 Inhibition of MMPs 23
1.2.3.1 TIMPs 23
1.2.3.2 Pharmacological inhibition of MMPs 23
1.2.4 MMPs and vascular function 24
1.2.5 MMPs and septic shock 27
1.2.5.1 Membrane microparticles in septic shock 29
1.2.5.2 Potential targets of MMPs in septic shock 30

1.3 Hypothesis and objectives 31

1.3.1 Matrix metalloproteinases contribute to lipopolysaccharide and interleukin-1β-induced vascular hyporeactivity (Chapter 3) 31
1.3.2 The endothelium contributes to LPS-induced vascular hyporeactivity (Chapter 4) 32
1.3.3 Matrix metalloproteinase inhibition *in vivo* protects against lipopolysaccharide-induced vascular hyporeactivity (Chapter 5) 33
1.3.4 Interleukin-1β-contributes to vascular hyporeactivity via a NO/cGMP dependent mechanism (Chapter 6) 33
1.3.5 Potential targets and novel pathophysiological mechanisms of MMPs in vascular hyporeactivity (Appendix: Additional Preliminary Studies) 34

CHAPTER 2
GENERAL METHODS

2.1 Animals 38
2.2 MMP inhibitors 38
2.3 Lipopolysaccharide-mediated loss of contractile tone in aortic rings *in vitro* 38
2.4 Interleukin-1β-induced vascular dysfunction *in vitro* 40
2.5 Rat endotoxemia 41
2.6 Assessment of vascular function of aortae taken from endotoxemic rats 41
2.7 Preparation of aorta homogenates 42
2.8 Determination of protein content 42
2.9 Measurement of MMP activity by zymography 42
2.10 Western immuoblotting analysis 43
2.11 Statistical analysis 44

CHAPTER 3
MATRIX METALLOPROTEINASES CONTRIBUTE TO LIPOPOLYSACCHARIDE AND INTERLEUKIN-1β-INDUCED VASCULAR HYPOREACTIVITY

3.1 Introduction 46
3.2 Methods 48
3.3 Results 52
3.4 Discussion 57

CHAPTER 4
ENDOTHELIAL DEPENDENCE OF MATRIX METALLOPROTEINASE-MEDIATED VASCULAR HYPOREACTIVITY CAUSED BY LIPOPOLYSACCHARIDE

4.1 Introduction 72
4.2 Materials and Methods 74
4.3 Results 75
4.4 Discussion 77

CHAPTER 5
INHIBITION OF MATRIX METALLOPROTEINASE ACTIVITY *IN VIVO* PROTECTS AGAINST VASCULAR HYPOREACTIVITY IN ENDOTOXEMIA

5.1 Introduction 85
5.2 Materials and methods 87
5.3 Results 90
5.4 Discussion 93

CHAPTER 6
THE NO/CGMP PATHWAY CONTRIBUTES TO MATRIX METALLOPROTEINASE-INDUCED VASCULAR HYPOREACTIVITY STIMULATED BY INTERLEUKIN-1β

6.1 Introduction 103
6.2 Materials and Methods 104
6.3 Results 106
6.4 Discussion 110

CHAPTER 7
DISCUSSION

7.1 Conclusions 129
7.2 Limitations 133
7.3 Future directions 135

APPENDIX I:
ADDITIONAL PRELIMINARY STUDIES

A1.1 Introduction: Membrane microparticles 140
A1.2 Methods 141
A1.3 Results 143
A1.4 Discussion 145

A2.1 Introduction: Calponin as a potential proteolytic target of MMP-2 146
A2.2 Materials and methods 149
A2.3 Results 150
A2.4 Discussion 151

A3.1 Introduction: Genetic ablation of MMP-2 151
A3.2 Materials and methods 152
A3.3 Results 152
A3.4 Discussion 153

CHAPTER 8
REFERENCES

8.1 Reference list 166

List of Tables

Table 5.1 pEC_{50} values of aortic contractile responses to PE.....96

Table 5.2 MMP activities in plasma using gelatin zymography.....101

Table 6.1 pEC_{50} values of aortic contractile responses to PE.....116

Table 6.2 pEC_{50} values of aortic contractile responses to PE.....122

Table 6.3 Contraction of IL-1β-treated aortae in response to 75 mM KCl.....123

Table 6.4 pEC_{50} values of aortic contractile responses to PE.....125

Table A1.1 Positive flow cytometric events of the microparticle fraction from the plasma of a control and a LPS-treated rat at 6 hr.....157

List of Figures

Figure 1.1 General structure of lipopolysaccharide from Gram-negative bacteria.....4

Figure 1.2 Simplified inflammatory pathway in lipopolysaccharide signalling.....6

Figure 1.3 Pathway for the generation of peroxynitrite and its toxic decomposition products.....17

Figure 1.4 Activation of matrix metalloproteinases via proteolysis and peroxynitrite.....22

Figure 1.5 Proteolytic regulation of vascular tone by matrix metalloproteinase-2....25

Figure 1.6 Central hypothesis diagram.....35

Figure 3.1 Structure of matrix metalloproteinase inhibitors doxycycline and GM6001.....63

Figure 3.2 Response of aortic rings taken from normal rats to a time-dependent loss of phenylephrine-induced tone.....64

Figure 3.3 Lipopolysaccharide-induced time-dependent loss of phenylephrine-induced tone in aortic rings taken from normal rats.....65

Figure 3.4 Responses of aortic rings taken from normal rats to interleukin-1β-induced vascular hyporeactivity.....66

Figure 3.5 Gelatinolytic and collagenolytic activities of homogenates prepared from aorta removed 6 hr after i.p. injection of lipopolysaccharide.....67

Figure 3.6 Gelatinolytic activities and immunoblots of matrix metalloproteinase-2 and -9 in aortic homogenate from lipopolysaccharide-treated rats.....68

Figure 3.7 Tissue inhibitor of metalloproteinase protein content in aortic homogenate from lipopolysaccharide-treated rats.....69

Figure 3.8 Contractile response of aortic rings taken from rats 6 hr after injection of lipopolysaccharide.....70

Figure 4.1 Nitric oxide synthase and matrix metalloproteinase dependence of lipopolysaccharide-induced vascular hyporeactivity.....81

Figure 4.2 GM6001 reduces lipopolysaccharide-induced vascular hyporeactivity.....82

Figure 4.3 Gelatinolytic activity of aortae stimulated with lipopolysaccharide ± doxycycline with or without the endothelium.....83

Figure 5.1 Concentration-response relationship to phenylephrine in aortae isolated from rats injected with lipopolysaccharide ± doxycycline.....97

Figure 5.2 Representative immunoblot for inducible nitric oxide synthase in aortic homogenate.....98

Figure 5.3 Gelatinolytic activity and immunoblot of matrix metalloproteinase-2 activity and protein, respectively.....99

Figure 5.4 Representative immunohistochemical distribution of matrix metalloproteinase-2 and -9 in aortic cross sections.....100

Figure 6.1 Contractile response of interleukin-1β-stimulated aortic rings incubated with or without matrix metalloproteinase inhibitors to increasing concentrations of phenylephrine.....115

Figure 6.2 Western blot of vascular homogenate inducible nitric oxide synthase protein levels.....117

Figure 6.3 Gelatinolytic activity and western blot of vascular homogenate matrix metalloproteinase-2 levels.....118

Figure 6.4 Zymogram of culture media from aortas incubated with interleukin-1β.....119

Figure 6.5 Effect of inhibitors of matrix metalloproteinase, nitric oxide synthase, and soluble guanylate cyclase on basal matrix metalloproteinase-2 activity, protein and release.....120

Figure 6.6 Contractile response of interleukin-1β-stimulated aortic rings incubated with or without inhibitors of nitric oxide synthase or soluble guanylate cyclase, to increasing concentrations of phenylephrine.....121

Figure 6.7 Contractile response of interleukin-1β-stimulated aortic rings incubated with or without a peroxynitrite decomposition catalyst or a superoxide dismutase mimetic.....124

Figure 6.8 Western blot of vascular homogenate matrix metalloproteinase-2 levels following treatment with interleukin-1β in the presence or absence of inhibitors of nitric oxide synthase or soluble guanylate cyclase.....126

Figure 6.9 Zymogram of culture media from aortas incubated with interleukin-1β in the presence or absence of inhibitors of nitric oxide synthase or soluble guanylate cyclase.....127

Figure A1.1 Two parameter dot plot histograms of staining by anti-annexin V-FITC in the microparticle fraction of plasma 154

Figure A1.2 Two parameter dot plot histograms of staining by anti-CD62E-Phyco in the microparticle fraction of plasma.....155

Figure A1.3 Two parameter dot plot histograms of staining by anti CD31-Alexafluor 647 in the microparticle fraction of plasma.....156

Figure A1.4 Concentration-response to phenylephrine of aortae incubated with the supernatant or microparticle fractions of the plasma.....158

Figure A1.5 Concentration-response to phenylephrine from aortae incubated with microparticle fractions of plasma in the presence or absence of GM6001.....159

Figure A1.6 Zymographic analysis of supernatant and microparticle fractions of plasma from control and lipopolysaccharide-treated rats.....160

Figure A2.1 Coomassie blue staining of troponin I and calponin degradation by matrix metalloproteinase-2 *in vitro*.....161

Figure A2.2 Calponin levels in aortic homogenates from control and lipopolysaccharide-treated rats.....162

Figure A2.3 Calponin levels in homogenates from aortae stimulated with interleukin-1β in the presence or absence of GM6001.....163

Figure A3.1 Concentration-response to phenylephrine in aortae from wild-type and matrix metalloproteinase-2 knockout mice.....164

List of Abbreviations

Alexa	alexafluor 647
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Cap	calponin
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
Doxy	doxycycline
DMSO	dimethyl sulfoxide
DQ	dequenched
Endo	endothelium
eNOS	endothelial nitric oxide synthase
E_{max}	maximal effect
EC_{50}	half maximal effective concentration
ET	endothelin
FeTTPS	5,10,15, 20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]
FITC	fluorescein isothiocyanate
GM6001	N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide
hr	hours

IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
K_{ATP}	adenosine triphosphate activated potassium channel
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
KO	knockout
L-NAME	N^G-nitro-L- arginine methyl ester
LPS	lipopolysaccharide
mg	milligram
µg	microgram
mL	millilitres
min	minutes
MMP	matrix metalloproteinase
MT-MMP	membrane type matrix metalloproteinase
MnTE-2-PyP	Manganese(III)-5,10,15,20-tetrakis(N-ethylpyridinium-2-yl)porphyrin pentachloride
MP	microparticle
NF-κB	nuclear factor kappa B
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

$OH^{\cdot}$	hydroxyl radical
$O_2^{\cdot-}$	superoxide
$ONOO^-$	peroxynitrite
ONO-4817	(2*S*, 4*S*)-*N*-hydroxy-5-ethoxymethyloxy-2-methyl-4-(4-phenoxybenzoyl)aminopentanamide
OPT	*o*-phenanthroline
PCR	polymerase chain reaction
PE	phenylephrine
Phyco	phycoerythrin
SEM	standard error of mean
sGC	soluble guanylate cyclase
SOD	superoxide dismutase
Std	standard
TIMP	tissue inhibitor of matrix metalloproteinase
TnI	troponin I
TNF-α	tumor necrosis factor alpha
v/v	volume by volume
w/v	weight by volume
WT	wild type

CHAPTER 1

INTRODUCTION

Portions of this chapter have been published in the following reviews:

Cena J., Chow, A.K., Schulz R. Nitric oxide, peroxynitrite, and matrix metalloproteinases: an insight into the pathogenesis of sepsis. In: Tota, B., Trimmer, B. (eds.), *Advances in Experimental Biology: Nitric Oxide*, 2007 (pp. 367-396). Amsterdam; Elsevier.

Chow, A.K., Cena J., Schulz R. Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. *Br J Pharmacol* 2007; 152:189-205.

In this introductory chapter I will review septic shock, the matrix metalloproteinases, and how these two subjects are linked. Section 1.1 will outline the epidemiology and pathophysiology of septic shock with particular emphasis on the vasculature. Section 1.2 introduces matrix metalloproteinases (MMPs) and their newly discovered roles in septic shock. Section 1.3 is comprised of the thesis hypotheses and objectives for each chapter.

1.1 Pathophysiology of septic shock

1.1.1 Definition of septic shock

Sepsis, a life-threatening condition arising through the body's response to an infection, is the leading cause of death in intensive care units in North America [1]. In the United States sepsis and its associated syndromes account for 2.9% of all hospital admissions and 10% of admissions into the intensive care unit [2]. In 1995, an estimated 9.3% of all deaths in the United States were attributed to sepsis. Mortality rates vary among the population, from 3.2% in children to 43% in the elderly. Additionally, the annual expenditure for sepsis in the US is estimated at $16.7 billion. In one specific health region in Canada, the mortality rate was 49% [3].

Sepsis is characterised by both an infection and a systemic inflammatory response [4]. Clinically, sepsis indicates what has also been termed 'severe sepsis' which is defined as sepsis complicated by global organ dysfunction. Septic shock, a subset of severe sepsis, is defined as sepsis manifested by circulatory failure and characterised by persistent arterial hypotension. Additionally, widespread intravascular coagulation

occurs, a result of downregulation of fibrinolytic mechanisms [5]. Research into the pathogenesis of sepsis has revealed many potential therapeutic targets such as inflammatory mediators, bacterial toxins, and specific enzymes; however, no completely effective pharmacotherapy has yet been discovered. Part of the problem in developing treatments for this syndrome is dealing with its enormous complexity [6].

Clinically, septic shock is typically characterized by fever, respiratory distress, tachycardia and persistent hypotension. Laboratory tests frequently show altered white blood cell counts, decreased platelet count and acidosis. Blood borne infection is confirmed by a positive blood culture, though the condition may progress to death before the results of the tests are available.

Treatment of septic shock typically requires admission into the intensive care unit. Therapy is mainly supportive: oxygen, fluid resuscitation, vasopressor and inotropic agents are applied in order to improve blood pressure and tissue perfusion. The effective tissue distribution of broad spectrum antibiotics applied intravenously to combat the infection is hindered by the persistent systemic hypotension. Consequently it is essential that the mechanisms that underlie the severe and persistent hypotension observed in septic patients become fully elucidated in order to facilitate more positive treatment outcomes. However, unravelling the underlying mechanisms is difficult because the natural immunological response to bacterial infection releases an unrelenting cascade of mediators, thus complicating the pathophysiology of sepsis.

1.1.2 Pathogenesis of septic shock

1.1.2.1 Lipopolysaccharide

Though the origin of bacterial infection can vary widely, the body's reaction to systemic invasion by bacterial pathogens follows a common cascade. Initiation of the septic shock cascade first begins with exposure to immunogens in the bloodstream. Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria (Figure 1.1), can spark the inflammatory cascade leading to sepsis-like conditions. LPS, also called

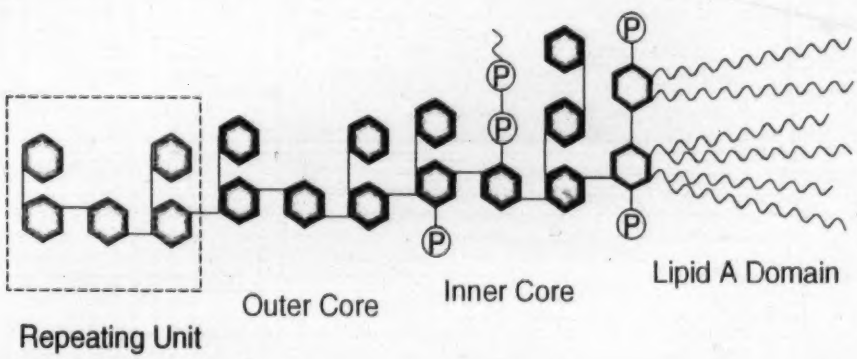


Figure 1.1 General structure of LPS from Gram-negative bacteria. LPS consists of a membrane-anchoring lipid A domain and a covalently linked polysaccharide portion. The terminal end of the LPS molecule contains up to 50 repeating oligosaccharide units. The central core domains are more structurally conserved among bacterial species.

endotoxin, consists of a membrane-anchored lipid A domain and a covalently linked polysaccharide portion [7]. The polysaccharide region consists of up to 50 repeating oligosaccharide units and has extreme structural variability among bacterial species. The terminal end of the LPS molecule, called the O-specific chain, protects the bacteria from phagocytosis. Interestingly, the polysaccharide moiety in gram-negative bacteria has evolved to mimic human glycolipids and thus allow them a consequential increased resistance to the immune system defenses. The lipid A domain is shown to be the primary

immunoreactive moiety of LPS. The immune system is highly sensitive to this domain, and administration of this domain alone can elicit immune responses [8]. LPS first binds to CD14 receptors in the immune system via its lipid A portion [9,10]. The LPS-CD14 complex is then recognized by toll-like receptor-4 (TLR-4) located on neutrophils, macrophages, and endothelial cells [11,12]. Occupancy of the TLR by the complex activates multiple signal transduction pathways and mobilizes transcription factors (such as nuclear factor-κB (NFκB) [13]) which stimulate transcription of many genes that encode for immunomodulatory molecules such as the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Many of these downstream mediators may stimulate the release of other factors potentially amplifying the inflammatory cascade. Accompanying the release of pro-inflammatory mediators is the release of anti-inflammatory cytokines such as transforming growth factor-β and others. In sepsis, an imbalance occurs which shifts the balance towards inflammation. Continuous generation of these mediators makes the pathogenesis of sepsis self-perpetuating and independent of the initial exposure to endotoxin.

Lipoteichoic acid and peptidoglycan are two major cell wall components in gram-positive bacteria, both of which stimulate inflammatory responses in various *in vitro* and *in vivo* models [14]. Approximately 50% of all cases of sepsis are initiated by gram-positive bacteria; *Staphylococcus aureus* being the most commonly associated microbe. Although there are many parallels between gram-positive and gram-negative sepsis and septic shock, this thesis will focus on the pathogenesis of gram-negative sepsis/septic shock and endotoxemia.

1.1.2.2 Pro-inflammatory cytokines

The inflammatory cascade in sepsis involves the generation of a plethora of powerful pro-inflammatory cytokines such as interleukin-2 (IL-2), IL-4, IL-6, IL-8, interferon-γ (IFN-γ), and TNF-α [15-19]. Release of this diverse array of cytokines complicates the search for the primary mediators involved in the inflammatory cascade in sepsis. However, recent evidence brings to light the importance of TNF-α and IL-1β in the inflammatory process (Figure 1.2). Natanson *et al.* (1988) have shown that in dogs

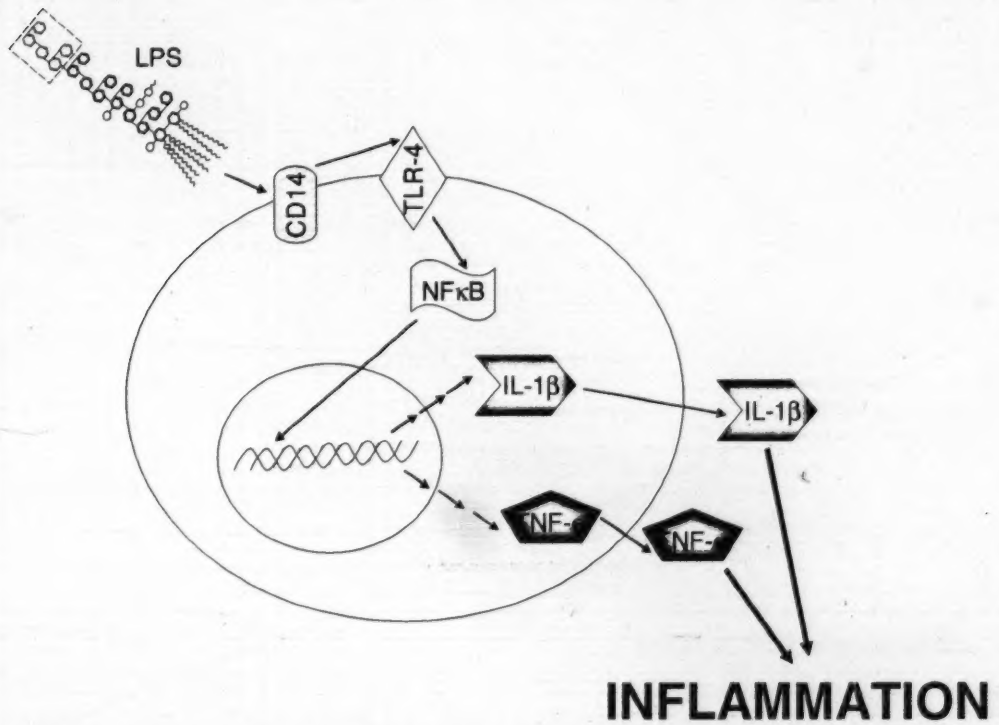


Figure 1.2 Simplified inflammatory pathway in LPS signalling. LPS stimulates intracellular pathways downstream of TLR4 via NFκB, leading to the transcription of pro-inflammatory cytokines such as IL-1β and TNF-α.

many cardiovascular manifestations of septic shock coincide with peak TNF-α serum concentrations [20]. Moreover, several groups have demonstrated a dose-dependent

correlation between the administration of TNF-α and the pathophysiological markers of septic shock in dogs [21-23]. Despite promising experimental evidence, clinical trials involving the blockade of TNF-α have been unsuccessful [24,25]. IL-1β is another important culprit of the inflammatory cascade in sepsis based on recent studies. Experimentally, infusion of this cytokine into animals results in cardiovascular dysfunction as well as other sepsis-related abnormalities [26,27]. Interestingly, blockade of IL-1β by receptor antagonists was found to reduce cardiovascular dysfunction and mortality in animal models [28,29]; however, human trials produced more modest results [30]. A possible explanation for the failure of these anti-cytokine trials may lie in the similarity of effects seen among the cytokine family. The redundancy of action observed in the cytokine cascade may compensate for the inhibition of any one particular cytokine, rendering inhibition of a specific pro-inflammatory cytokine an ineffective therapeutic strategy.

Therapies involving the inhibition of cytokine synthesis or their mechanism of action may be detrimental. Animal studies demonstrate that antibody neutralization of TNF-α activity increases mortality, and combination therapy (blockade of both TNF-α and IL-1β receptors) enhanced fatality in various models of sepsis [31,32]. Possible reasons for these results may be that suppression of the immune system allows pathogenic substances to exert their effects unhindered, and some suggest that clinical trials involving immunosuppressive agents were inappropriately conducted [33]. A crucial problem concerning these failed clinical trials appears to be the heterogeneity of the patient population involved. The categorization of septic patients enrolled in these trials exhibited discrepancies leading to unreliable or misleading conclusions [34].

1.1.3 Vascular manifestations of septic shock

The development of persistent systemic vasodilation in sepsis involves two interacting factors. The first is the enhanced biosynthesis of vasodilatory substances from the endothelium [35] and from other physiological sources. The second involves the central theme of this thesis, hyporeactivity of vascular smooth muscle to vasoconstriction.

1.1.3.1 Vascular hyporeactivity

Normally, endogenous adrenergic agonists such as noradrenaline maintain vascular tone by occupying α-receptors on vascular smooth muscle. Very high doses of vasoconstrictors which would cause severe hypertension in the healthy individual are necessary in the septic patient in order to at least partially reverse the marked hypotension [36]. This suggests that the vasculature is insensitive to endogenous and exogenous vasoconstriction.

Although the cellular pathogenesis of vascular hyporeactivity is unclear, several theories have been presented. The cytokines are now considered to be a central factor in the pathogenesis of vascular hyporeactivity. TNF-α, IL-1β, and IL-6 are three proinflammatory cytokines that are important mediators of hyporeactivity [37]. It has also been suggested that superoxide ($O_2^{\cdot-}$) produced by activated neutrophils and macrophages and associated with tissue damage and the inflammatory response, can oxidize catecholamines and render them incapable of effecting vasoconstriction; therefore, benefits of such vasoconstrictor agents are limited [38]. There is emerging evidence which implicates a disruption in ion homeostasis across the vascular smooth muscle cell membrane in sepsis. Chen *et al.,* (2005) demonstrated an abnormal activation of the

Na^+/K^+ ATPase pump in aortae from endotoxemic rats [39]. This results in a rapid depletion of cellular energy as well as a decrease in contractility due to the ionic imbalance. The ATP-sensitive potassium channel (K_{ATP}) has also been implicated in the pathogenesis of vascular hyporeactivity. Studies have demonstrated an increase in K_{ATP} channel activity in the vascular smooth muscle of LPS-treated rats [40]. This results in vascular smooth muscle cell hyperpolarization, thereby limiting the contractile response to vasoconstrictors.

1.1.3.2 Role of the endothelium in septic shock

The vascular endothelium lines the luminal side of blood vessels and serves as an interface between the circulating blood and tissue. It performs a variety of physiological functions which are essential for homeostasis. These include the regulation of organ perfusion, vascular tone and permeability, as well as the adhesion of platelets and blood-borne cells and the modulation of coagulation [41]. By virtue of its contact with the bloodstream, the endothelium is first to come in contact with pathogens. A large body of evidence supports the role of the endothelium in orchestrating the immune response in sepsis [42,43]. Endothelial activation leads to the release of vasodilators allowing immune cells to infiltrate the infected tissue. Under normal conditions, the endothelium functions as an anticoagulant surface; however, during sepsis it is believed to undertake a procoagulatory phenotype [42] by expressing tissue factors leading to the activation of Factor VII [44,45] and consequential activation of the extrinsic coagulation pathway.

The decrease in vascular tone observed can be explained by enhanced production of several vasodilatory substances. Cytokines such as TNF-α, IFN-γ and IL-1β have been

shown to produce excessive amounts of two potent vasodilators, prostaglandin I_2 and NO (see below) [35]. These vasodilators may be endothelial-derived or result from increased biosynthesis in vascular smooth muscle [46,47].

1.1.3.3 Models of vascular hyporeactivity

1.1.3.3.1 Lipopolysaccharide-induced vascular hyporeactivity *in vitro*

Clinically, septic shock is associated with marked vascular hyporeactivity to vasoconstrictors [35]. This phenomenon can be mimicked in an *in vitro* experimental setting in which LPS is applied directly onto blood vessel preparations [48-50]. Both clinical septic shock and *in vitro* LPS administration share parallels with respect to their mechanisms of pathogenesis. Both are associated with an overproduction of NO and an excessive activation of soluble guanylate cyclase (sGC) [51,52]. Furthermore, application of LPS *in vitro* results in the generation of pro-inflammatory cytokines which are important effectors in septic shock [53,54]. Utilization of *in vitro* models of vascular hyporeactivity induced by LPS has revealed potential mediators in the development of this phenomenon including NO and sGC described above [55,56]. These studies have revealed other targets such as protein kinase C and vascular K^+ channels as potential mediators of vascular hyporeactivity in models of septic shock. Knowledge gained from pharmacological investigations utilizing that model has led to various clinical trials in septic shock; these include the use of NOS or K^+-channel blockade. [57-64]. LPS-induced vascular hyporeactivity *in vitro* provides a system with limited confounding variables and the capability to experimentally design parallel functional and biochemical analysis from the same tissue. One of the main advantages is that it differentitates

between direct versus indirect vascular effects of LPS. This design generates more scientifically relevant data because the functional changes are directly reflected in the biochemical analysis.

1.1.3.3.2 Lipopolysaccharide-induced vascular hyporeactivity *in vivo*

The model of septic shock involving the injection of LPS *in vivo* in lab animals is commonly known as endotoxemia. It is defined as the presence of LPS (endotoxin) in the plasma, which has also been examined in human volunteers [65-67]. The value of this model in animals is related to its ease of reproducibility and manifestation of septic shock symptoms including inflammation, overproduction of vasodilatory substances, and vascular hyporeactivity to vasoconstrictors; moreover it elicits a similar cytokine profile that occurs in septic shock [53,54,68].

In studying vascular hyporeactivity, many groups have utilized this model to gain information on the mechanisms involved in the consequences of endotoxemia [40,69-75]. Fundamentally, as an *in vivo* experimental system, endotoxemia is a biologically relevant model of septic shock and has established this significance in various studies examining vascular hyporeactivity [59,69,76]. In those studies, a model of rat endotoxemia was utilized in order to investigate the involvement of NOS or K^+-channels which are believed to contribute to vascular hyporeactivity. These studies were further developed into clinical trials examining the impact of these phenomena [61,64].

1.1.3.3.3 Interleukin-1β-induced vascular hyporeactivity *in vitro*

There is a well-established role of pro-inflammatory cytokines in the development of cardiovascular dysregulation seen in septic shock. IL-1β has emerged as a key mediator in the development of vascular hyporeactivity to vasoconstrictors [77], and symptomatically produce vascular manifestations similar to those seen in clinical septic shock. IL-1β has been shown to have effects distinct from LPS which contribute to vascular hyporeactivity. For example LPS is capable of stimulating L-arginine transport in cultured vascular smooth muscle cells, whereas IL-1β is not [78]. In another cell culture model, examination of the effects of LPS and IL-1β revealed different G-protein signalling mechanisms with respect to NO production and iNOS protein synthesis [79]. In this model, inhibitors of G-protein signalling potentiated the effects of LPS but not IL-1β-induced generation of NO and stimulation of iNOS protein synthesis. *In vivo*, marked differences exist in the cardiovascular changes that occur when comparing LPS and IL-1β infusion into rats [27]. Specifically, the recovery in mean arterial pressure was much slower in LPS versus cytokine treated rats; moreover, this recovery in mean arterial pressure was lower in LPS-treated rats.

More importantly, our enzymes of interest, the matrix metalloproteinases (MMPs), have been shown to regulate the biological activity of various cytokines [80]. For example, MMP-2 and -9 have been shown to proteolytically activate the IL-1β precursor into its biologically active form [81]. Therefore, we utilized an *in vitro* model of IL-1β downstream of this activation in order to understand the mechanisms resulting in the pathological activation of this pro-inflammatory cytokine on vascular hyporeactivity. Additionally, tetracyclines, a class of antibiotics which have been recently discovered to

be potent inhibitors of MMPs, have been demonstrated to decrease levels of IL-1β in the plasma in mouse models of septic shock [82].

1.1.4 Molecular mechanisms of pathogeneity

1.1.4.1 Nitric oxide

Since the discovery of NO, scientific research has unravelled its biosynthesis and roles in many physiological systems. Moreover, NO has been a central component of various cardiovascular diseases including septic shock. NO is upregulated during sepsis and overproduction of this free radical is a well established indicator of, and contributor to, the pathogenesis of septic shock.

NO is a small, short-lived molecule that has received tremendous attention in recent years. It is a labile gas with a half-life in the time frame of seconds in physiological solution at 37 °C [83-85]. The effects of NO were first observed in aortic rings; light mechanical rubbing of the lumen of a segment of rabbit aorta (a protocol for removing the vascular endothelium) completely inhibited the vasorelaxant properties of acetylcholine [86]. At that time, the chemical identity of this relaxant factor was unknown and it was termed endothelium-derived relaxing factor (EDRF). It was later determined to be NO [87,88]. The generation of NO is catalyzed by three distinct isoforms of NOS, endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). All isoforms of NOS catalyze the oxidation of the amino acid L-arginine to produce NO and citrulline. NO is primarily generated in the cardiovascular system by a Ca^{2+}-dependent NOS in cardiac myocytes [65], endocardial endothelial cells [89], and vascular endothelial cells [90]. This NOS isoform was later identified to be eNOS [91].

nNOS activity has also been localized to the sarcoplasmic reticulum in cardiac myocytes [92], as well as to cardiac neurons [93,94] and blood vessels innervated with non-adrenergic non-cholinergic nerves where it is released as a neurotransmitter [95]. NO exerts a number of regulatory and cytoprotective effects such as promoting vasodilation [85], and decreasing the adhesion of platelets [96] and neutrophils [97] to the endothelium, as well as inhibiting platelet aggregation [98].

Oxidation of one of the guanidine nitrogen atoms of L-arginine forms NO and citrulline. In this process, 1.5 molecules of NADPH per molecule of NO are consumed and molecular oxygen is reduced [99]. NO exerts many but not all of its biological effects via its direct activation of sGC which catalyzes the formation of cGMP from guanidine triphosphate [100]. sGC contains a haem moiety which is essential for the binding of NO and the activation of the enzyme [101]. cGMP can be acted upon by phosphodiesterases which render it biologically inactive. Of the phosphodiesterase family, phosphodiesterase V is mainly responsible for the enzymatic cleavage of cGMP in vascular smooth muscle cells [102].

The main downstream action of cGMP generation is the activation of protein kinase G, its associated protein kinase. This kinase is involved in the regulation of various enzymes via phosphorylation [103]. In vascular smooth muscle, this ultimately promotes vasodilation via Ca^{2+}-dependent and independent mechanisms. Specifically, protein kinase G acts to decrease myosin phosphorylation by inhibition of Rho A as well as activate K^+ channels and inhibit the inositol triphosphate receptor [104].

Under inflammatory conditions, NO can be produced in higher concentrations following the expression of iNOS in the endocardial endothelium [105], vascular

endothelial cells [106], cardiac myocytes [65,107], vascular smooth muscle [108], and neutrophils [109]. Pro-inflammatory cytokines such as those involved in the inflammatory cascade in sepsis (eg. TNF-α, IL-1β and IFN-γ) are capable of inducing iNOS [110] in these cell types. This isoform of NOS continuously produces NO at high concentrations which can be sustained over several hours, attributed to its ability to catalyze NO formation in a Ca^{2+}/calmodulin-independent manner [111] in the presence of sufficient amount of L-arginine substrate and other NOS cofactors [112].

Evidence of enhanced NO and/or peroxynitrite ($ONOO^-$, see below) production is found in the plasma of septic patients as their metabolites, NO_2^- and NO_3^- [113,114]. This overproduction of NO contributes to cardiac dysfunction and systemic vasodilation. This deleterious combination imparts a fatal decrease in cardiac output resulting in an impairment of tissue perfusion and oxygen extraction [115]. The potential relevance of NO in these conditions was supported by *in vivo* animal studies demonstrating the attenuation of cardiovascular effects of cytokines and LPS by NOS inhibitors [76,116,117].

During endotoxemia or sepsis, pro-inflammatory cytokines stimulate the expression of iNOS in a variety of cell types including endothelial cells, macrophages, Kupffer cells, cardiac myocytes and vascular smooth muscle cells [65,108,118,119]. Vascular smooth muscle, for example, is capable of expressing iNOS and this is believed to be responsible for the excessive production of NO and subsequent vasodilation seen in sepsis [119]. Additionally excessive production of NO by iNOS has been shown to be cytotoxic to the endothelium [108,120]. iNOS knockout mice are resistant to endotoxin-induced hypotension, indicating the involvement of this NOS isoform in the pathogenesis

of sepsis [121]. However, various animal studies and clinical trials involving the specific pharmacological inhibition of NOS have revealed difficulties in this strategy [122-124]. This may be due to the variable induction of iNOS at different anatomical sites. Excessive inhibition of NOS may result in augmented microvascular vasoconstriction, cell hypoxia and lactic acidosis [125,126]. Moreover, it is evident that NO also plays some protective roles in the setting of septic shock and that a careful titration of NOS inhibitor is required to only partially block excess NO production in this setting [124,127]. These observations bring to light a possible therapeutic strategy targeting the inhibition of iNOS induction, however, as previously mentioned, clinical trials designed to block various inflammatory mediators participating in iNOS induction have produced only modest results [25].

1.1.4.2 Oxidative stress

A considerable body of evidence suggests that enhanced oxidative stress is a major contributor to endotoxic shock. Moreover, antioxidant therapy has proven beneficial in various models of sepsis. A key link between NO and oxidative stress in sepsis is the discovery of $ONOO^-$ as a mediator in this process. Oxidative stress results from an imbalance between oxidant and antioxidant species. It is believed to be a central component of and contributor to various cardiovascular pathologies, including septic shock (Figure 1.3). H_2O_2, $O_2^{\cdot-}$, hydroxyl radical ($OH^{\cdot}$), and $ONOO^-$ represent the best known reactive oxygen species. $O_2^{\cdot-}$ is normally reduced to H_2O_2 via superoxide dismutase (SOD) [128] and the H_2O_2 is then metabolized intracellularly by either

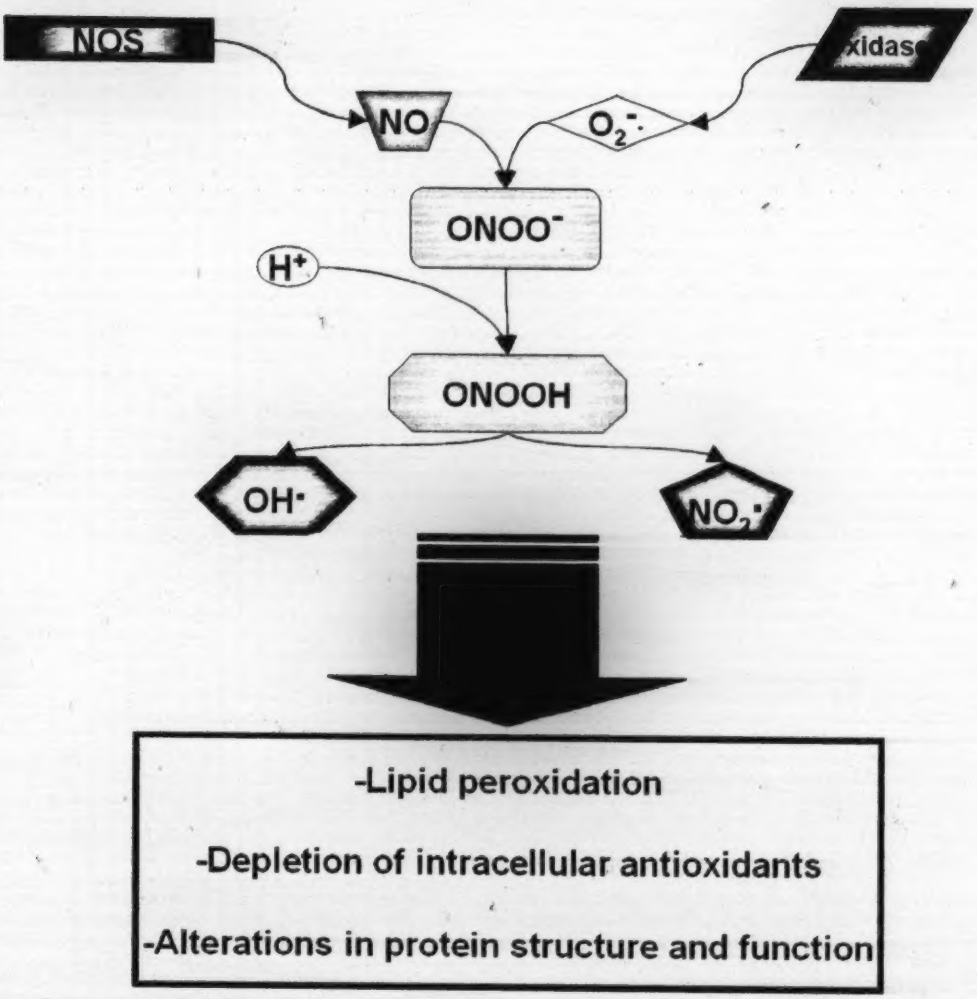


Figure 1.3 Pathway for the generation of peroxynitrite and its toxic decomposition products. Excess NO can combine with O_2^{-} to form $ONOO^-$. At physiological pH $ONOO^-$ can become protonated and spontaneously decompose into the toxic metabolites OH and NO_2.

glutathione peroxidise or catalase. However, in some scenarios it decomposes to OH via the iron-dependent Fenton reaction [129]. Both O_2^{-} and OH are free radicals, the latter is several orders of magnitude more reactive than the former [130,131]. These radicals initiate a chain reaction particularly with membrane lipids, further perpetuating their damaging effects. NADPH oxidase and xanthine oxidase, two major sources of O_2^{-}, are

also increased in the vascular wall [132] and heart [66,133] during endotoxemia or exposure of these tissues to pro-inflammatory cytokines.

The mechanism by which NO exerts damaging effects throughout the cardiovascular system involves the formation of $ONOO^-$, the toxic reaction product of NO and $O_2^{\cdot-}$ [134]. During sepsis, the upregulation of NO biosynthesis coincides with an increase in the generation of $O_2^{\cdot-}$. At physiological pH, $ONOO^-$ is protonated to form the unstable intermediate peroxynitrous acid. This then readily decomposes into several products including the highly reactive free radicals, nitrogen dioxide ($NO_2^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$).

Previous studies have confirmed the involvement of $ONOO^-$ in human and in animal models of sepsis by a variety of experimental methods [66,135,136]. Activation of poly (ADP-ribose) polymerase (PARP) by $ONOO^-$ has emerged as a significant mechanism in the reduction of cardiac contractility and vascular hyporeactivity in sepsis [137,138]. PARP is a highly conserved enzyme found in nuclei which is involved in the repair of single strand DNA breaks by recruiting and activating DNA repair enzymes. Increased PARP activation during sepsis, however, can impart a disturbance in cellular metabolism by depleting intracellular stores of its substrate, nicotinamide adenine dinucleotide (NAD^+) [139]. This results impaired glycolysis, Krebs' cycle activity, and mitochondrial electron transport, thus resulting in adenosine triphosphate (ATP) depletion. PARP inhibitor treatment reduced the vascular hyporeactivity to vasoconstrictors as observed in the aortae of LPS-treated rats [138]. PARP activation is also involved in the regulation of various inflammatory proteins including iNOS as well as other cytokines and chemokines [140].

1.2 Matrix metalloproteinases

Recent evidence indicates that $ONOO^-$ as well as various cytokines can enhance the activity and/or expression of matrix metalloproteinases (MMPs), targeting them as important culprits for the cardiovascular dysfunction associated with sepsis. A growing body of evidence supports the involvement of MMPs as well as the beneficial effects of MMP inhibition in various models of sepsis.

1.2.1 Classification and structure

MMPs are a large family of zinc dependent endopeptidases which were first discovered as a collagenolytic activity released from the tail of a tadpole undergoing metamorphosis [141]. They are best known as proteolytic enzymes which degrade extracellular matrix proteins, including collagen, necessary for tissue remodelling processes. MMPs are classified by numerical designation (MMP-1 through MMP-28) and are also categorized by their *in vitro* substrate specificity for certain extracellular matrix substrates. Groups of MMPs include the collagenases (MMPs -1, -8, and -13) stromelysins (MMP-3 and -10), matrilysins (MMP-7 and -26) membrane-type MMPs (MT-MMPs, 1 through 8), and the gelatinases (MMP-2 and -9).

All MMPs are initially synthesized in an inactive zymogen form ("proMMP"). Structurally, MMPs have a signalling peptide at the N-terminus allowing for secretion into the endoplasmic reticulum and eventual transport out of the cell. Beside the signal peptide lies a hydrophobic propeptide domain involved in shielding the catalytic domain next to it. This catalytic domain is present in all MMPs and is known as the 'matrixin fold' which forms substrate binding pockets. The catalytic Zn^{2+} is coordinated to a

cysteinyl sulphydryl group on the propeptide domain. This intermolecular association is termed the 'cysteine switch' and is conserved across the MMP family [142].

The key feature of the collagenases is their ability to cleave a specific site of interstitial collagens I, II, and III [143]. The stromelysins can activate a number of pro-MMPs including proMMP-1 [144]. Matrilysins are characterized by the lack of an intact hemopexin domain [143]. Membrane-type MMPs are transmembrane and glycosyl phosphatidyl inositol anchored proteins. They are all capable of activating proMMP-2 as well as digesting a number of extracellular matrix substrates. The gelatinases are known for their ability to digest denatured collagens (gelatin). These enzymes have three repeats of a type II fibronectin domain contained within the catalytic domain which bind gelatin, collagens, and laminin [145]. Those MMPs not classified into the above categories are involved in other biological functions including macrophage migration (MMP-12) [146], enamel formation (MMP-20) [147], and in repairing epidermal tissue [148].

The propeptide domain must be perturbed in order for the MMP to become proteolytically active. Various mechanisms have been elucidated concerning activation of this class of enzymes and each is distinct in its own way. One mechanism involves the activation of MMPs in the extracellular space which occurs in two distinct steps. First, activation is initiated by other proteases (eg. trypsin or other MMPs) that cleave the propeptide at specific sites. Upon cleavage, the shielding of the catalytic cleft is withdrawn, exposing the catalytic Zn^{2+} ion. The propeptide, however, is not entirely removed and the newly active MMP undergoes intermolecular autocatalysis which cleaves the remaining propeptide thus generating a lower molecular weight enzyme [149]. MMPs can also be activated by other members of its class. One important example

involves the activation of MMP-2. MT1-MMP, which is located in the plasma membrane, associates with two molecules of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). This complex binds pro-MMP-2 allowing for its proteolytic activation of MMP-2 [150]. Another mechanism of activation involves the intracellular protease, furin [151-153]. In contrast to extracellular activation, this action takes place intracellularly as furin is present in the Golgi apparatus. However, the proteolytic action of furin on MMP-2 in the Golgi can also result in an inactive MMP-2 [154]. After proteolytic activation, the MMP is either targeted to the cell membrane for insertion (in the case of MT-MMPs) or secreted from the cell.

1.2.2 Regulation and function

Interestingly, activation of MMPs can occur both intracellularly and extracellularly via a direct post-translational modification of a cysteine residue in the auto-inhibitory propeptide domain (Figure 1.4). In this pathway, oxidative species such as H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$, HOCl [155] and $ONOO^-$ oxidize the sulphydryl bond of the cysteinyl group involved in coordinating the catalytic Zn^{2+} ion. This causes a conformational change in the enzyme which exposes the catalytic Zn^{2+} ion and produces an active enzyme to which the propeptide domain is still attached [156-158]. There is inaccuracy in the current nomenclature of MMPs in that proMMPs are commonly referred to as the inactive zymogen form of MMPs solely due to their higher molecular weight, as seen in SDS-PAGE. Whereas MMP activation by oxidative stress results in a proteolytically active "proMMP" form which is only distinguishable in molecular weight from its zymogen form with mass spectrometry, and not SDS-PAGE.

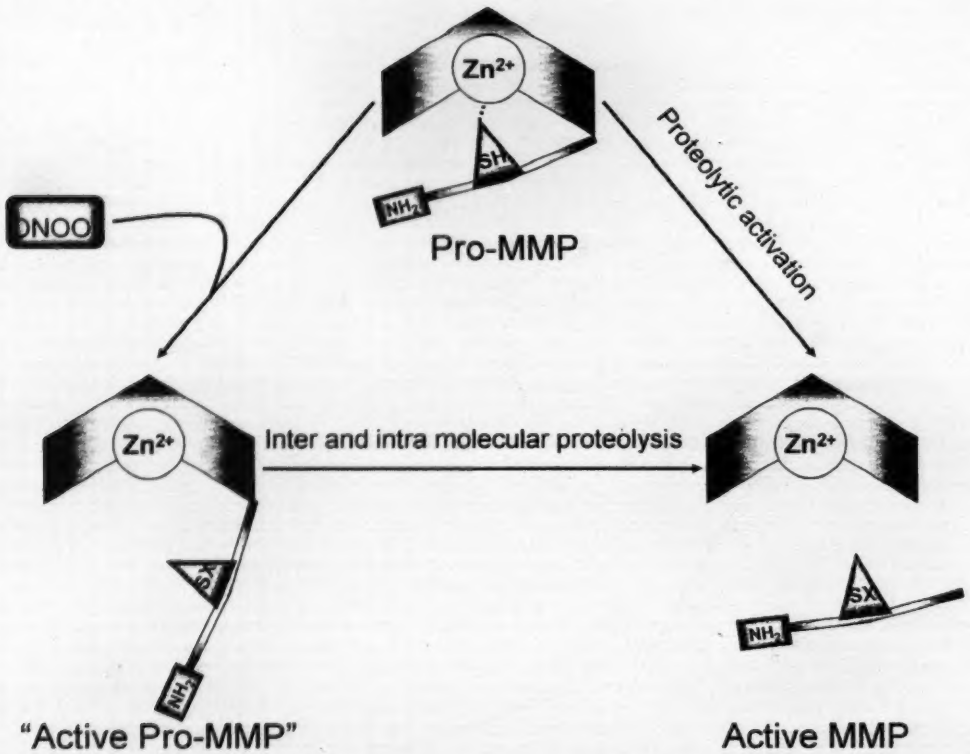


Figure 1.4 Activation of MMPs via proteolysis and $ONOO^-$. MMPs are synthesized as inactive zymogens (pro-MMP) and are classically thought to be activated via proteolytic removal of the propeptide by other proteases including MMPs (right pathway). However, under conditions of oxidative stress, $ONOO^-$ is capable of oxidizing the cysteinyl sulphydryl (designated as 'SH') coordinated to the catalytic Zn^{2+} ion. This "active pro-MMP" can cleave susceptible protein targets and can also undergo inter and intramolecular catalysis to yield an active MMP without the propeptide domain.

Despite the large variability in function, MMPs are best known to degrade extracellular matrix proteins and are involved in both physiological and pathological processes including embryogenesis, organogenesis, angiogenesis, wound healing, and platelet aggregation [159,160]. The MMPs have been implicated in the pathogenesis of cancer, inflammatory arthritis, and pulmonary and cardiovascular diseases [80,161-163].

1.2.3 Inhibition of MMPs

1.2.3.1 TIMPs

The tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous proteins involved in the regulation and inhibition of MMP activity. To date four TIMPs have been identified and each binds to a MMP in a 1:1 stoichiometric ratio [164]. Structurally, TIMPs have an N-terminal inhibitory domain and a smaller C-terminal domain. TIMPs 1-4 have a broad range of inhibitory activity against several MMPs. These TIMPs differ in that TIMP-2 is constitutively expressed whereas TIMP-1 can be induced by pro-inflammatory cytokines [165]. TIMP-3 is less characterized but has been shown to be involved in angiogenesis as well as lung abnormalities [166]. In the heart, TIMP-3 is found in the extracellular matrix [167,168]. TIMP-4 is perhaps the most widespread TIMP in the cardiovascular system and has been localized to the sarcomere of cardiac myocytes [169]. It is suggested to have cardioprotective effects [170].

1.2.3.2 Pharmacological inhibition of MMPs

Pharmacological inhibitors of MMP activity exhibit different mechanisms of action. The tetracycline antibiotics are recognized to have MMP inhibitory activity distinct from their antimicrobial effects [171]. Doxycycline is the most potent MMP inhibitor of the tetracycline class of antibiotics and has been shown to cross cell membranes [173] and exhibits MMP inhibitory action at plasma concentrations lower than those required for its antimicrobial effect [174]. Upon examination of MMP-7, a MMP without a hemopexin domain, doxycycline was also shown to interact with structural Ca^{2+} and Zn^{2+} ions as an inhibitory mechanism [175]. GM6001 is a hydroxamic

acid based MMP inhibitor designed to act as a bidentate ligand for the catalytic Zn^{2+} in the active site [172]. Pharmaceutical companies produced over 50 compounds which inhibit MMPs activity as promising treatments for inflammatory diseases (eg. rheumatoid arthritis) and cancer; however, only one has been approved, a sub-antimicrobial dose formulation of doxycycline (Periostat®) for periodontal inflammation [176].

1.2.4 MMPs and vascular function

Proteolytically active MMPs are capable of generating vasoactive peptides from numerous substrates. MMP-2 cleaves big endothelin-1 (big ET-1) generating an active vasoconstrictor peptide, medium ET-1, which is a more potent vasoconstrictor than ET-1 itself [177]. Additionally, MMP-2 was also found to cleave calcitonin gene-related peptide, thus abolishing its vasodilatory capacity [178]. Moreover, MMP-2 cleaves the vasodilatory peptide adrenomedullin resulting in the generation of both vasodilatory and vasoconstrictive products [179]. Thus, through its proteolytic action on vasoactive peptides and their precursors, MMP-2 has effects on the control of vascular tone (Figure 1.5). MMP-2 and -9 have vasodilatory properties in vascular smooth muscle via inhibition of Ca^{2+} entry mechanisms [180]. In these experiments, addition of recombinant MMP-2 and -9 to isolated rat aortae contracted with phenylephrine or KCl

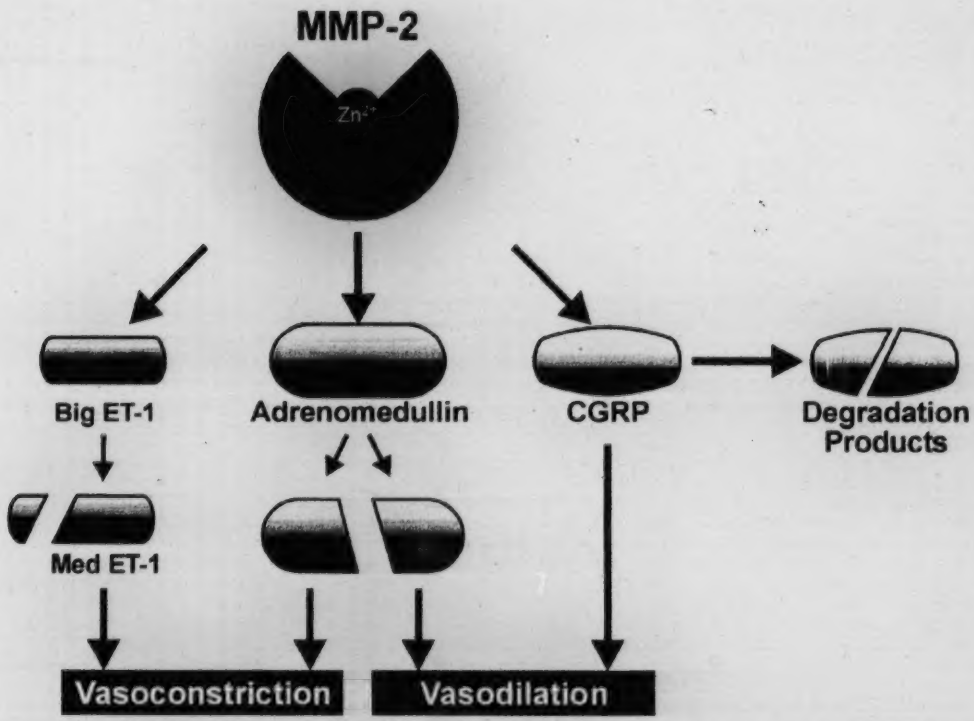


Figure 1.5 Proteolytic regulation of vascular tone by MMP-2. MMP-2 can regulate vascular tone via proteolysis of vasoactive peptides. Left: MMP-2 cleaves big endothelin-1 (Big ET-1) to yield a potent vasoconstrictor, medium endothelin-1 (Med ET-1). Centre: MMP-2 can also proteolyze the vasodilatory peptide adrenomedullin resulting in the generation of both vasoconstrictor and vasodilator peptides. Right: MMP-2 is also able to cleave calcitonin gene-related peptide (CGRP) to non-vasoactive degradation products, thus inactivating its vasodilatory activity.

resulted in vasorelaxation. The effect of the MMPs appears to be independent of actions on the extracellular matrix as no histological changes in the extracellular matrix were detected. The same group observed an upregulation of K^+-channel activity and subsequent hyperpolarization in the rat inferior vena cava upon exogenous MMP-2 application [181]. Literature exists demonstrating a relationship between vascular relaxation due to decreases in intracellular Ca^{2+} and MMP-mediated actions on integrins [182,183]. Specifically, $\alpha_v\beta_3$, an integrin expressed by vascular smooth muscle may

interact with arginine-glycine-aspartic acid (RGD) peptides which can be generated by MMP-mediated cleavage of extracellular matrix components. These peptides have been shown to exhibit vasodilatory capabilities via reducing intracellular Ca^{2+} levels [184]. Another possibility is the activation of endothelial protease-activated receptors via their cleavage by thrombin or trypsin which have been shown to initiate NO-mediated vascular relaxation [185]. MMP-2 also upregulates plasma membrane associated Ca^{2+}-ATPase activity in pulmonary vascular smooth muscle [186]. Interestingly, this effect of MMP-2 is synergistic in combination with oxidizing agents such as H_2O_2 [187]. Together, these observations suggest a vasodilatory role for MMPs via alterations in Ca^{2+} handling. A caveat of these studies is that MMPs often co-purify with TIMPs and thus the latter may represent a significant contaminant in MMP preparations. Thus whether some of these effects are due to TIMP activity remains to be tested.

MMPs are also involved in regulating vascular tone via signal transduction pathways. In a study examining small arteries in rats, the upregulation of MMP-9 activity was discovered to contribute to the vasodilatory effects of relaxin [188]. In this study, relaxin induced MMP-9 activity after 4-6 hr exposure in mesenteric blood vessels. Agonists of various G-protein coupled receptors have also been shown to cause vasoconstriction through MMP-7 activation in rat mesenteric arteries [189]. Specifically this involves the activation of epidermal growth factor receptors and mitochondrial reactive oxygen species [189,190].

Interestingly, hypoxia results in enhanced MMP-2 activity in murine aortae and mesenteric arteries which is shown to promote vasoconstriction [191]. Although these

studies demonstrate the strong involvement of MMPs, the proteolytic targets of MMP action have yet to be elucidated.

1.2.5 MMPs and septic shock

Although few studies have dealt with the relationship between MMPs and sepsis, strong evidence linking the two has been provided by researchers using isolated cell culture models, animal models, and in clinical studies with septic patients. The focus has centered on the gelatinases (MMP-2 and -9) since they are either abundant (MMP-2) or can be induced by cytokines (MMP-9) in a variety of cardiovascular cell types. Xie *et al.*, (1994) demonstrated the concentration-dependent relationship between LPS and the stimulation of MMP-2 and -9 activities in isolated murine macrophages [192]. Pugin *et al.*, (1999) performed similar experiments using human blood [193]. Blood MMP-9 activity was increased after stimulation with LPS. Albert *et al.*, (2003) found that circulating MMP-9 activity increased significantly within 2 hr after administration of LPS to human volunteers [194]. These studies, among others, demonstrate the relationship between LPS and MMP-2 and –9 activities providing a possible link between MMPs and clinical sepsis.

In baboons subjected to *E. coli*-induced sepsis, increased MMP-9 activity was found in serum [195]. Dubois *et al.*, (2002) conducted a study using MMP-9 knockout mice and found that they were significantly more resistant to lethal doses of LPS as compared to the control mice [196]. Carney *et al.*, (2001) studied the utility of MMP inhibition using a chemically modified tetracycline (devoid of antibacterial activity, yet

retaining MMP inhibitory action) in pigs which were administered LPS [197]. The LPS treated group exhibited a dramatic reduction in blood pressure and lung function which was abolished in the group treated with a MMP inhibitor, demonstrating the involvement of MMPs in the development of severe hypotension and lung injury induced by LPS. Steinberg *et al.,* (2005) showed that the same chemically-modified tetracycline significantly reduced morbidity and prevented the reduction of blood pressure in a pig model of septic shock induced by the introduction of a fecal blood clot into the peritoneal cavity and occlusion of the mesenteric artery [198]. Lalu *et al.* (2003) demonstrated that the MMP inhibitors, Ro 31-9790 or doxycycline, attenuated LPS-induced myocardial contractile dysfunction in rats. In the same rat model of endotoxemia [73], Lalu *et al.,* (2004) found that the symptoms of endotoxic shock in rats which include lethargy, piloerection and porphyrin secretion from the eyes peaked 6-12 hr after LPS administration and were accompanied by a subsequent loss in ventricular MMP-2 activity [75]. Likewise, plasma levels of MMP-2 were found to be significantly depressed 3-12 hr post-LPS administration. Plasma MMP-9 activity and protein levels, however, peaked 1 hr following LPS administration. The balance between MMPs and TIMPs is an important factor to consider in dealing with MMP activity. One study by Martin *et al.* (2003) demonstrated that TIMP-3 knockout mice are more prone to the detrimental effects of sepsis on pulmonary airway compliance due to the increased activities of MMP-2 and -9 [199].

Myocardial depression in sepsis ultimately involves a reversible attenuation of the contractile efficiency of the myofilaments in cardiac myocytes [35]. This may be due to decreased cytosolic Ca^{2+} release, dysregulation of intracellular Ca^{2+} and/or decreased

myofilament sensitivity to Ca^{2+}. These mechanisms have been shown using *in vivo* models of sepsis-induced myocardial dysfunction [1]. Indeed, activation of MMP-2 occurs in cytokine-induced myocardial depression along with the concomitant degradation of key contractile elements in the cardiac sarcomere such as troponin I [200].

Although cytokines have been shown to upregulate MMP activity and expression, they are in fact regulated by MMPs themselves [80]. Certain members of the MMP family have been shown to be involved in the proteolytic processing of both pro- and anti-inflammatory cytokines and chemokines; MMP-2 and -9 were shown to process pro-IL-1β into its biological active form [81]. Zhang *et al.* (2003) revealed a mechanism in which the chemokine stromal cell derived factor-1 is cleaved by MMP-2 into a highly neurotoxic substance [201]. Conversely, MMP-2 mediated cleavage of monocyte chemoattractant protein-3 results in a product which is an antagonist of the receptors for this protein, thus providing a mechanism by which a MMP may attenuate inflammation [202]. These complexities add both a new dimension and possible cautions at the frontier of MMP drug design.

1.2.5.1 Membrane microparticles in septic shock

Membrane microparticles are shed from the plasma membrane of various cell origins including platelets, leukocytes, and endothelial cells. These MMP-containing vesicles [203] are increased in number following stimulation by cytokines or LPS in various *in vitro* models [204]. Recent evidence implicates membrane microparticles in the development of various cardiovascular inflammatory conditions including preeclampsia and septic shock [205-209]. Interestingly, microparticles of endothelial

origin have been implicated in the development of vascular hyporeactivity in preeclampsia [206,210-212]. Therefore, it is logical to assume a possible role of microparticles in the development of vascular hyporeactivity in septic shock.

1.2.5.2 Potential targets of MMPs in septic shock

Because the association between MMPs and septic shock has only recently been investigated, the pathophysiological targets of these enzymes have yet to be discovered. Our laboratory has implicated an intracellular role for MMP-2 in myocardial ischemia/reperfusion injury [213] and in cytokine-mediated cardiac contractile dysfunction [200]. In these studies, MMP-2-mediated degradation of troponin I was shown to impair cardiac contractile function during reperfusion following ischemia. Thus far a possible intracellular target of MMP-2 in smooth muscle has not been identified. Calponin is a 34 kDa actin-binding protein that is homologous to troponin I [214] and is located in the cytoskeleton and the contractile apparatus of smooth muscle cells [215]. Therefore, it is logical to hypothesize its susceptibility to MMP-2 degradation in pathological conditions. Moreover, calponin is susceptible to cleavage by calpain [216], an intracellular cysteine protease; and, pharmacological inhibitors of this enzyme (ie. calpastatin) have been shown to inhibit MMPs (unpublished data, Schulz lab). Indeed there appears to be an overlap in intracellular substrates susceptible to cleavage by either MMP-2 or calpain, particularly in cardiac myocytes [213,217].

1.3 Hypothesis and objectives

In this thesis, the association of MMPs and vascular hyporeactivity to vasoconstrictors in various models of septic shock will be investigated. Chapter 3 introduces this relationship by utilizing three distinct models of vascular hyporeactivity: LPS-induced vascular hyporeactivity *in vitro*, LPS-induced vascular hyporeactivity *in vivo*, and IL-1β-induced vascular hyporeactivity *in vitro*. Chapter 4 examines the role of the endothelium in LPS-induced vascular hyporeactivity *in vitro*. In order to investigate the biological relevance of these findings, LPS was administered *in vivo* in the presence of the MMP inhibitor, doxycycline; this relationship was examined in Chapter 5. Because factors produced by the immune system play an important role in the pathogenesis of vascular hyporeactivity to vasoconstrictors, a model of IL-1β-induced vascular hyporeactivity was studied in further detail in Chapter 6. A summary of these hypotheses is depicted in Figure 1.6. The Appendix briefly explores potential targets of MMPs as well as novel pathophysiological mechanisms for the development of vascular hyporeactivity to vasoconstrictors.

1.3.1 Matrix metalloproteinases contribute to lipopolysaccharide and interleukin 1β-induced vascular hyporeactivity (Chapter 3)

Although there have been a few studies examining the beneficial effect of pharmacological or genetic ablation of MMPs in septic shock, no studies have examined the role of MMPs in vascular hyporeactivity to vasoconstrictors in models of septic shock. The objectives in this chapter are to examine and characterize a possible role of MMPs in three models of septic shock.

Hypothesis 1.3.1.1 *MMPs contribute to vascular hyporeactivity in various models of endotoxic shock.*

Hypothesis 1.3.1.2 *MMP activity is upregulated in aortae of rats injected with LPS in vivo.*

Hypothesis 1.3.1.3 *Inhibition of MMPs in vitro protects against LPS and IL-1β-induced vascular hyporeactivity.*

1.3.2 The endothelium contributes to LPS-induced vascular hyporeactivity (Chapter 4)

The endothelium is in integral component of the vasculature and is involved in vasoregulation as well as orchestrating immune responses. Therefore, in this chapter I investigated the dependence of MMP-induced vascular hyporeactivity stimulated by LPS in the presence and absence of the endothelium in an *in vitro* model of LPS-induced vascular hyporeactivity.

Hypothesis 1.3.2.1 *MMP activity is upregulated in aortae treated with LPS in vitro.*

Hypothesis 1.3.2.2 *The endothelium contributes to LPS-induced vascular hyporeactivity.*

Hypothesis 1.3.2.3 *There is a differential protective action of MMP inhibition in LPS-treated aortae in the presence or absence of the endothelium.*

1.3.3 Matrix metalloproteinase inhibition *in vivo* protects against lipopolysaccharide-induced vascular hyporeactivity (Chapter 5)

Although several studies have examined the beneficial effects of pharmacological inhibition of MMPs in various models of septic shock, no study has examined the effect of MMP inhibition on LPS-induced vascular hyporeactivity to vasoconstrictors *ex vivo*. I therefore examined the contractile responses of rat aortae *ex vivo* following *in vivo* administration of LPS with or without the MMP-inhibitor, doxycycline.

Hypothesis 1.3.3.1	*MMP is upregulated in the aortae and plasma of endotoxemic rats.*
Hypothesis 1.3.3.2	*iNOS protein upregulation stimulated by LPS is unaltered following doxycycline treatment.*
Hypothesis 1.3.3.3	*Injection of the MMP inhibitor doxycycline in vivo prevents against LPS-induced vascular hyporeactivity to vasoconstrictors ex vivo.*

1.3.4 Interleukin-1β-contributes to vascular hyporeactivity via a NO/cGMP dependent mechanism (Chapter 6)

Although models of septic shock include administration of LPS *in vitro* and *in vivo,* pro-inflammatory cytokines including IL-1β have been demonstrated to contribute to vascular hyporeactivity to vasoconstrictors. Moreover, LPS and IL-1β exhibit distinct mechanisms of action. NO has emerged as a central mediator in the vascular hyporeactivity to vasoconstrictors; therefore I decided to examine potential downstream

targets of NO and their relationship to MMP regulation and contribution to vascular hyporeactivity.

Hypothesis 1.3.4.1 *MMPs are activated in the aorta in response to IL-1β which contributes to vascular hyporeactivity.*

Hypothesis 1.3.4.2 *MMP-induced vascular hyporeactivity stimulated by IL-1β is NO/cGMP dependent.*

1.3.5 Potential targets and novel pathophysiological mechanisms of MMPs in vascular hyporeactivity (Appendix: Additional Preliminary Studies)

This thesis has established both a relationship between MMPs and vascular hyporeactivity to vasoconstrictors induced by LPS, and several mechanisms of pathogeneity; however, the potential targets of MMPs within the vasculature during endotoxic shock are currently unknown. In the Appendix I explored a possible interaction of MMP-2 with calponin, an intracellular regulator of smooth muscle contraction. Our laboratory has demonstrated an intracellular pathophysiological role of MMP-2 in the heart via cleavage of troponin I, a regulator of cardiac myocyte contraction; therefore it was logical to begin the search for intracellular targets in vascular smooth muscle by investigating proteins homologous to troponin. Here, we examine the susceptibility of calponin to proteolysis by MMP-2 as well as the biological significance of this cleavage.

The generation of microparticles containing MMP activity is associated with clinical septic shock and moreover, these entities are also capable of inducing vascular hyporeactivity to vasoconstrictors. I therefore examined the possibility whether

membrane microparticles play a pathophysiological role in the development of vascular hyporeactivity to vasoconstrictors in endotoxemic rats.

Hypothesis 1.3.5.1	*MMP-2 cleaves calponin in vitro.*
Hypothesis 1.3.5.2	*Calponin levels are altered in the aorta of rats in various models of septic shock.*
Hypothesis 1.3.5.3	*Membrane microparticles are upregulated in endotoxemia and contribute to vascular hyporeactivity.*

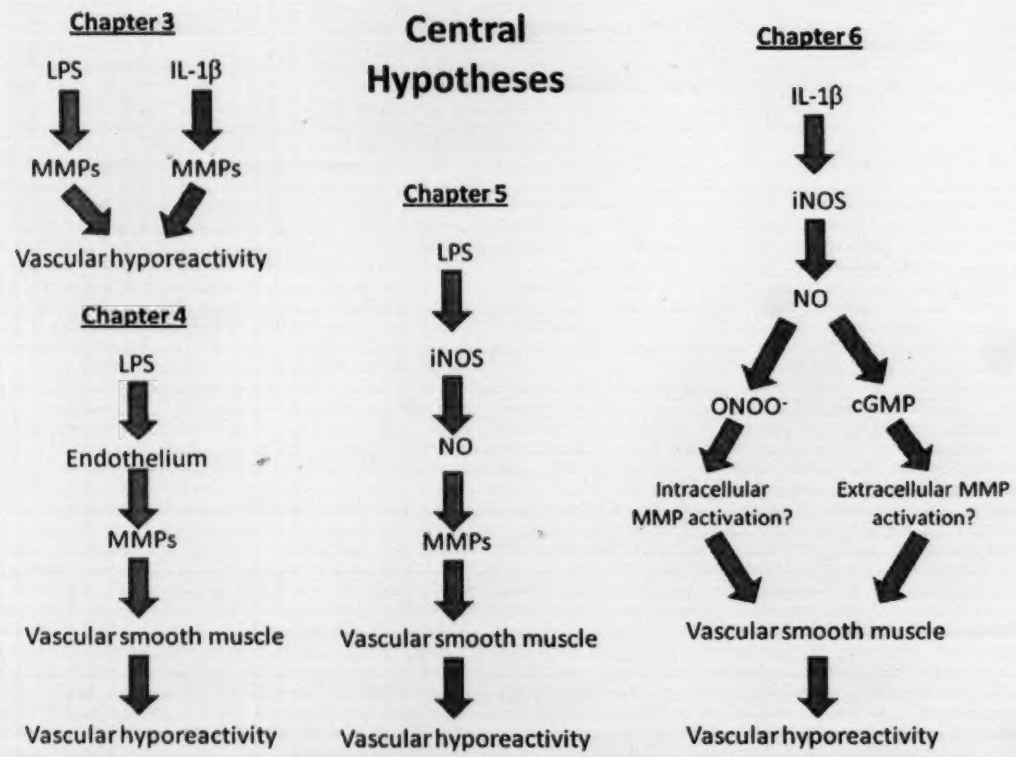


Figure 1.6 Central hypotheses diagram. Top-left: Hypothesis for Chapter 3, LPS and IL-1β contribute to vascular hyporeactivity via an MMP-dependent mechanism. Bottom-left: Hypothesis for Chapter 4, LPS upregulates MMP activity via an endothelium-dependent mechanism which contributes to vascular hyporeactivity. Middle: Hypothesis for Chapter 5, LPS contributes to vascular hyporeactivity *in vivo* via a MMP-dependent mechanism that is downstream of iNOS. Right: Hypothesis for Chapter 6, IL-1β contributes to MMP-induced vascular hyporeactivity via the generation of $ONOO^-$ and/or cGMP.

CHAPTER 2

GENERAL METHODS

2.1 Animals

This investigation conforms to the *Guide to the Care and Use of Laboratory Animals* published by the Canadian Council on Animal Care (revised 1993). Male Sprague Dawley rats (250-350 g) were used in all experiments.

2.2 MMP inhibitors

Doxycycline hyclate (Sigma) and GM6001 (Chemicon), two chemically distinct MMP inhibitors, were used in these studies. Doxycycline is the most potent of the tetracycline class of antibiotics and is recognized to have MMP inhibitory activity distinct from its antimicrobial effects [171]. Specifically, doxycycline has been shown to interact with structural Zn^{2+} ions of MMPs [175]. GM6001 is a hydroxamic acid based MMP inhibitor designed to act as a bidentate ligand for the catalytic Zn^{2+} in the active site [172]. ONO-4817, an MMP inhibitor more selective for MMP-2 and -9 versus other MMPs [218] was obtained from ONO Pharmaceuticals (Japan).

2.3 Lipopolysaccharide-mediated loss of contractile tone in aortic rings *in vitro*

Rats were killed by sodium pentobarbital overdose (100 mg/kg, i.p.). Thoracic aortae were rapidly excised and connective tissue was trimmed away in gassed (95% O_2-5% CO_2) Krebs solution (118 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7\ H_2O$, 2.5 mM $CaCl_2 \cdot 2\ H_2O$, 11.1 mM D-glucose, 25 mM $NaHCO_3$) bubbled with carbogen at room temperature. One to three aortic rings (5 mm in length) were

dissected from each animal. The rings were then mounted in organ baths filled with Krebs buffer at 37 °C which was continuously bubbled with 95% O_2-5% CO_2. Isometric tension was measured using force transducers (Grass FT03) and recorded using AcqKnowledge 3.1 software. A tension of 1 g was applied and the rings were equilibrated for 60 min with the Krebs buffer replenished at 20 min intervals. Following equilibration, LPS (300 ng/mL, *Salmonella typhosa*, Sigma) was added. Aortae were contracted with phenylephrine (750 nM, Sigma) and at the plateau of contraction one of the following was added: the MMP inhibitors doxycycline (30 μM) or GM6001 (30 μM), or their vehicles (doxycycline: ddH_2O; GM6001: 100% ethanol or DMSO, as indicated in appropriate chapter). Vascular tone was then monitored for 6 hr. This model was modified from a previously used model of vascular hyporeactivity [108].

In order to determine if LPS affects the generation of active tension, aortae were incubated in organ baths at 1 g tension for 6 hr with or without LPS. A submaximal dose of PE (750 nM) was applied and the contractile response was measured. After washing, resting tension was changed to 0.5 g and 2.0 g and aortae were equilibrated for 10 min before the contractile response to PE was examined for each. In control aortae, there was a significant increase in the generation of active tension at 2.0 g versus 1.0 g and 0.5 g of passive tension. In LPS-treated aortae, there was a significant increase in the generation of active tension at 2.0 g versus 0.5 g. These results are consistent with another study examining the effects of LPS treatment *in vivo* on the generation of active tension in the rat aorta which revealed an increased sensitivity to vasoconstrictors at higher levels of passive tension [219].

2.4 Interleukin-1β-induced vascular dysfunction *in vitro*

Isolated aortae were washed three times in sterile phosphate buffered saline (2.7 mM KCl, 1.47 mM KH_2PO_4, 136.7 mM NaCl,8 mM $Na_2HPO_4 \cdot H_2O$) supplemented with an antibiotic cocktail (100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL gentamicin, Sigma), and then dissected under a tissue culture hood. Rings were cut (5 mm in length) and then washed three times in phosphate buffered saline before a final wash in Dulbecco's modified Eagle's medium (with 1000 mg/L glucose, pyroxidine HCl, $NaHCO_3$; supplemented with antibiotic cocktail, Sigma). Rings were then placed in fresh Dulbecco's modified Eagle's medium (750 μL) with one or more of the following added: IL-1β (10 ng/mL, R & D Systems), GM6001 (10 or 30 μM), or GM6001 vehicle (100% ethanol or DMSO, as indicated in appropriate chapter). Rings were then incubated for 6 hr at 37 °C in a humidified atmosphere containing 5% CO_2. The rings were removed from the medium and mounted in organ baths as described above. The culture medium was sampled for MMP activity by zymography immediately to avoid freeze-thaw cycles which would affect MMP activity (see below). Following equilibration (60 min) under 1 g of tension, a concentration-response curve to phenylephrine (1 nM – 10 μM) was obtained. Rings were then washed with Krebs solution and the maximum contractile response to KCl (75 mM) was determined.

2.5 Rat endotoxemia

Rats were given either a non-lethal dose of LPS (*Salmonella typhosa*, Sigma, 4 mg/kg i.p.) or pyrogen-free water (control). Rats were then killed with sodium pentobarbital overdose (100 mg/kg, i.p.) at 6 hr. Previous investigations have revealed that in this model of endotoxemia, NO production is increased by this time point, and that cardiovascular function is significantly depressed [65,73]. Aortae were rapidly excised and connective tissue was trimmed away in carbogen-gassed Krebs solution. After trimming, the aortae were cut into 5 mm sections and either processed for functional analysis or blotted to remove excess water and rapidly frozen in liquid nitrogen and stored at -80 °C for later processing.

2.6 Assessment of vascular function of aortae taken from endotoxemic rats

Aortae were isolated after 6 hr from either LPS or vehicle-treated rats as above, and two 5 mm rings from each animal were mounted in organ baths. Following equilibration and washes with Krebs buffer (4 times at 15 min intervals) while under 1 g of tension, a concentration-response curve to phenylephrine (1 nM – 10 μM) was obtained in order to confirm that vessels from LPS-treated animals were hypocontractile compared to vessels from control animals.

2.7 Preparation of aorta homogenates

Frozen aortae were crushed using a stainless steel piston that was cooled to liquid N_2 temperature. The resulting powder was diluted 1:4 w/v in 50 mM Tris-HCl (pH 7.4) buffer containing 3.1 mM sucrose, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 0.1% Triton X-100, and protease inhibitor cocktail (P8340, Sigma). This solution was then homogenized by hand on ice using a motorized pellet pestle (Kontes). The homogenate was centrifuged at 10 000 g for 5 min at 4 °C and the supernatant was kept on ice for biochemical analysis.

2.8 Determination of protein content

Aortic homogenate protein content was determined by the bicinchoninic acid method (Sigma) using bovine serum albumin as a standard.

2.9 Measurement of MMP activity by zymography

Gelatinolytic activities of MMPs were examined by gelatin zymography as previously described [220,221]. 8% polyacrylamide gels copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma) were prepared. Non-heated samples were diluted with water in order to load a constant amount of protein per lane (10 μg of protein from aorta incubation media, 20 μg of protein from aorta homogenate). A standard was loaded into one lane of each gel (supernatant of phorbol ester activated HT-1080 cells, American Type Culture Collection) to determine the molecular weights of gelatinolytic

activities as well as to serve as an internal standard used to normalize activities between gels. Following 1.5 hr of electrophoresis under a constant voltage of 150 V, the gels were washed with 2.5% Triton X-100 for 1 hr at room temperature (with three changes of solution) to remove sodium dodecyl sulphate. Gels were then incubated for 20 to 30 hr at 37 °C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3). After incubation the gels were stained with 0.05% Coomassie Brilliant Blue (G-250, Sigma) in a mixture of methanol:acetic acid:water (2.5:1:6.5, v/v) and destained in aqueous 4% methanol:8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark blue background. Zymograms were digitally scanned and band intensities were quantified using ImageJ (National Institutes of Health Research, version 1.36b) and expressed as a ratio to the HT-1080 internal standard. In order to confirm that quantified gelatinolytic activities were of MMP origin, addition of either *o*-phenanthroline (100 μM), GM6001 (10 μM), or doxycycline (30 μM) to the incubation buffer was found to abolish all gelatinolytic activities.

2.10 Western analysis

Aorta homogenate (10 – 20 μg protein) was loaded onto (8 – 12%) polyacrylamide gels, electrophoresed at a constant voltage of 150 V under reducing conditions, and then electroblotted onto polyvinylidene difluoride membranes (BioRad) in Towbin buffer (20% v/v methanol, 25 mM Tris-base, 192 mM glycine, 0.05% w/v sodium dodecyl sulphate). Positive standards and/or molecular weight standards (Precision plus protein standard (dual color), 161-0374, Biorad) were also loaded into gels in order to confirm the identity of proteins to be probed. Polyvinylidene fluoride

membranes were permeabilized by soaking in methanol for 1 min. Proteins were transferred onto polyvinylidene fluoride membranes at 100 V for 1 hr. Following transfer, membranes were blocked in 5% w/v skim milk powder in TTBS buffer (0.001% v/v Tween-20, 2 M Tris (pH 7.6), 0.10 M NaCl) for 2 hr at room temperature or overnight at 4 °C. After blocking, membranes were incubated in 5 mL of 5% w/v skim milk powder in TTBS buffer with appropriate primary antibodies as described in each chapter; membranes were either incubated for 2 hr at room temperature or overnight at 4 °C. After incubation, membranes were washed with TTBS 3 times at 5 min intervals. Blots were subsequently incubated with the appropriate horseradish peroxidase conjugated antibodies (either anti-mouse or anti-rabbit, Transduction Laboratories) in 10 mL of 5% w/v skim milk powder in TTBS buffer for 1 hr at room temperature. After incubation, membranes were washed with TTBS buffer 5 times at 5 min intervals. Membranes were visualized using the horseradish peroxidase-luminol chemiluminesence reaction kit (Amersham Pharmacia Biotech).

2.11 Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) for *n* animals or homogenates as indicated in each chapter. The results were analyzed by using GraphPad Prism version 4.0. Independent samples t-test, repeated measures two-way ANOVA followed by Bonferroni's post hoc test, or one way ANOVA followed by Neuman-Keuls post hoc test were used as indicated to evaluate differences between groups. Differences were considered significant at $*p < 0.05$.

CHAPTER 3
MATRIX METALLOPROTEINASES CONTRIBUTE TO LIPOPOLYSACCHARIDE AND INTERLEUKIN-1β-INDUCED VASCULAR HYPOREACTIVITY

A portion of this chapter has been published. Lalu, M.M.*, Cena, J.*, Chowdhury, R.*, Lam, A., Schulz, R. Matrix metalloproteinases contribute to endotoxin and interleukin-1β induced vascular dysfunction. *Br J Pharmacol.* 142: 31-42, 2006. * denotes equal co-first authors.

3.1 Introduction

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases that are key regulators of the extracellular matrix. The gelatinases, MMP-2 and MMP-9, contribute to a wide variety of chronic cardiovascular pathologies including heart failure, atherosclerosis, and abdominal aneurysms [159,222]. Alterations in the levels of their main endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs), may also play a role in these chronic pathologies.

Recently, MMP-2 has been implicated in a number of acute cardiovascular processes, such as myocardial ischemia-reperfusion injury [169,213,220,223] regulation of normal vascular tone [224-226], platelet aggregation [160], and modulation of the inflammatory response [227]. Since MMPs are involved in inflammation and control of vascular tone, we hypothesized that they may be involved in the vascular alterations that occur in septic shock.

Septic shock is a potentially fatal condition in which a systemic bacterial infection produces an unencumbered inflammatory response. This inflammatory response then results in excessive vasodilatation, hyporeactivity to contractile agents, and cardiac dysfunction. It is the chief cause of death and disability in intensive care units [228]. A similar response and cardiovascular dysfunction can be provoked by administering endotoxin (lipopolysaccharide, LPS) to animals or human volunteers [67,229,229]. In both sepsis and endotoxemia the initial inflammatory response due to LPS is largely mediated by proinflammatory cytokines (including interleukin-1β (IL-1β) [230]) and tumor necrosis factor-α [231,232] which increase the production of a number of downstream effectors. One effector is peroxynitrite [233], the toxic reaction product of

nitric oxide [108] and superoxide anion [234] which are produced in excess during severe acute inflammation. Peroxynitrite biosynthesis is enhanced in aortae from LPS-treated rats [72]. It reacts with a number of different proteins [235] and lipids [236] to cause structural damage, enzyme dysfunction, and eventually cell death. Interestingly, both proinflammatory cytokines and peroxynitrite increase MMP activity and decrease TIMP activity *in vitro* [157,237,238]. Thus it was speculated that an imbalance between MMPs and TIMPs occurs during acute inflammatory stress.

To date, few investigations have examined the role of MMPs in septic shock or endotoxemia. In experiments using endothelial cells, neutrophils, or macrophages, LPS was found to increase MMP-2 and MMP-9 activities [192,193,239]. In both animal and human models of endotoxemia, circulating MMP-9 activity is increased [75,193-195]. In sepsis, circulating MMP-2 and MMP-9 are elevated in both animal models and patients [195,240,241]. Despite these insights, no study has investigated the functional role of MMPs in the vascular dysfunction that arises from severe, acute inflammatory stress.

In order to address this issue, we employed three previously established models of vascular inflammatory stress: either LPS or IL-1β stimulation of aortae isolated from normal rats or aorta isolated from rats administered LPS intraperitoneally. Both functional, pharmacological, and biochemical approaches were taken to determine changes in vascular contractility and the MMPs and TIMPs in the vessel wall.

3.2 Methods

Spontaneous loss of contractile tone in aortic rings

Two models of LPS-induced vascular hyporeactivity were utilized in this chapter. The first method involving ambient LPS-mediated vascular hyporeactivity is described here. Untreated rats were killed by sodium pentobarbital overdose (100 mg/kg, i.p.). Aortae were rapidly excised and connective tissue was trimmed away in gassed (95% O_2-5% CO_2) Krebs buffer (118 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7\ H_2O$, 2.5 mM $CaCl_2 \cdot 2\ H_2O$, 11.1 mM D-glucose, 25 mM $NaHCO_3$) bubbled with carbogen at room temperature. One to three aortic rings (5 mm in length) were dissected from each animal. If more than one ring was prepared from one rat, the individual rings were used in different experimental groups. The rings were then mounted in organ baths filled with Krebs buffer at 37 °C which was continuously bubbled with 95% O_2-5% CO_2. Isometric tension was measured using force transducers (Grass FT03) and recorded using AcqKnowledge 3.1 software. A tension of 1 g was applied and the rings were equilibrated for 60 min with fresh Krebs buffer added at intervals of 20 min. Following equilibration, rings were contracted with phenylephrine (750 nM, Sigma). At the plateau of contraction one of the following was added: polymyxin B (10 μg/mL, Sigma), a drug which binds and inactivates LPS [242], or the MMP inhibitors (Figure 3.1) doxycycline (30 μM), GM6001 (10 or 30 μM), or their vehicles (doxycycline: ddH_2O, GM6001: 0.1% ethanol in ddH_2O). Vascular tone was then monitored for another 4.5 hr. This model has been previously used as a model of vascular hyporeactivity caused by the presence of ambient levels of LPS in experimental

environments in which LPS is not controlled [107]. The second model utilizing LPS-induced vascular hyporeactivity is described in Section 2.3.

IL-1β induced vascular dysfunction

Two models of IL-1β-induced vascular dysfunction were utilized. First, aortae were isolated and contracted with phenylephrine (750 μM) as described above in the organ bath. At the plateau of contraction, human recombinant IL-1β (10 ng/mL, R&D Systems) was added in the presence of either doxycycline (30 or 100 μM) or its vehicle (ddH_2O). Vascular tone was then monitored for 6 hr and the final tension was reported.

The second model involving IL-1β-induced vascular hyporeactivity *in vitro* (where aortic rings are incubated with IL-1β in cell culture medium prior to mounting them in organ baths) is described in Section 2.4.

Rat endotoxemia

This protocol is described in Section 2.5.

Assessment of vascular function of aortae taken from endotoxemic rats

Vessels were prepared as described in Section 2.5 and subjected to the following protocol: after contractile measurements, aortae were then washed and incubated with either doxycycline (100 μM, Sigma) or ddH_2O vehicle for 10 min. Following incubation, all vessels were subject to a second concentration-response curve to phenyephrine (1 nM – 10 μM). Vessels were then washed and the maximal contractile response to KCl (75 mM) was determined.

Determination of plasma nitrate/nitrite levels

In both sets of rats a blood sample was drawn from the chest cavity immediately upon sacrifice. The plasma fraction was obtained following centrifugation (6500 g for 5

min at 4 °C) and stored at -20 °C for later determination of plasma nitrite and nitrate levels. Plasma was diluted 1:1 with deionized water and then deproteinized by centrifugal ultrafiltration (Ultrafree-MC microcentrifuge tubes UFC3, Millipore). Ultrafiltrates were analyzed for total nitrate and nitrite content according to the method of Green *et al* [243].

Preparation of aorta homogenates

This protocol is described in Section 2.7.

Determination of protein content

This protocol is described in Section 2.8.

Gelatinase and collagenase assays

In order to measure the net activity of gelatinases (MMP-2 and MMP-9), aortic homogenate (100 μg of protein) was analyzed using a gelatinase assay kit (E-12055, Molecular Probes). Non-activated samples were incubated at room temperature in the presence of DQ gelatin, an internally quenched fluorescein conjugate that fluoresces upon cleavage. Digestion of this product yields fluorescent peptides that are detectable using a fluorometer (λ_{ex} 495 nm, λ_{em} 515 nm). The MMP inhibitor *o*-phenanthroline (100 μM) was added to duplicate samples in order to determine MMP-related gelatinase activity. Pretreament of aortic homogenate from a control rat with *p*-aminophenylmercuric acetate, a synthetic chemical activator of metalloproteinases, increased gelatinolytic activity approximately tenfold.

In order to measure the activities of collagenases (MMP-1, -8, and -13), aortic homogenates (50 μg of protein) were analyzed using an MMP collagenase assay kit (ECM710, Chemicon) according to the manufacturer's instructions. The samples, however, were not chemically treated to activate latent collagenase activity. Biotinylated

collagen was digested by collagenase activity in the samples at 37 °C. The biotinylated fragments were then transferred to a biotin binding 96 well plate and detected with a streptavidin-enzyme complex. In order to control for baseline collagen degradation a series of wells were loaded with only biotinylated collagen and no sample. Addition of a colorimetric substrate produced a colored reaction product which was detectable at 450 nm. Addition of 10 μM GM6001 abolished all collagenase activity.

Measurement of MMP activity by zymography

This protocol is described in Section 2.9.

Immunoblot analysis

The protocol is described in Section 2.10 using the following primary antibodies: an anti-mouse monoclonal MMP-2 antibody (1:1000 dilution, MAB3308, Chemicon), a rabbit polyclonal anti-rat MMP-9 antibody which detects the 92 kDa form of this protein (1:4000 dilution, courtesy of Dr. Mieczyslaw Wozniak, Medical University, Wroclaw, Poland), a mouse monoclonal anti-human TIMP-1 antibody (2 μg/mL, MS-608, NeoMarkers), a rabbit polyclonal anti-human TIMP-2 antibody (10 μg/mL dilution, RB-1489, NeoMarkers), a mouse monoclonal anti-human TIMP-3 (1 μg/mL, 136-13H4, Calbiochem) or a rabbit polyclonal anti-human TIMP-4 antibody (0.2 μg/mL, AB19087, Chemicon).

Statistical Analysis

This is described in Section 2.11.

3.3 Results

MMP inhibition ameliorates spontaneous loss of phenylephrine induced vascular tone

In order to assess whether MMP inhibition affects LPS mediated vascular dysfunction, aortae from normal rats were mounted in organ baths, contracted with phenylephrine, and their tone was then monitored for 6 hr. A spontaneous and significant loss of vascular tone at the end of the observation period was noted in these contracted vessels (to 58 ± 4% of original phenylephrine-induced tone (Figure 3.2)). This spontaneous loss of tone has previously been demonstrated to be due to ambient levels of LPS [107]. This was confirmed in our experimental conditions by abolishing the spontaneous loss of tone with polymyxin B (10 μg/mL), a drug that binds and inactivates LPS (Figure 3.2A). In order to evaluate the role of MMPs in the spontaneous loss of contractile tone, two pharmacologically distinct MMP inhibitors were tested. Doxycycline (30 μM) or GM6001 (10 μM) abolished the spontaneous loss in vascular tone (Figure 3.2B and C).

MMP inhibition ameliorates LPS-induced loss of phenylephrine-induced vascular tone

In contrast to the spontaneous loss of phenylephrine-induced tone described above, in a new series of experiments exogenous LPS (300 ng/mL, *Salmonella typhosa*) was added before phenylephrine for a more reproducible and controlled model of vascular hyporeactivity. In the presence of added LPS, a significant loss of vascular tone occurred at the end of 6 hr (30 ± 4% of original phenylephrine-induced tone, Figure 3.3). In order to evaluate the role of MMPs in this model, doxycycline (30 μM) or GM6001

(30 μM) were added simultaneously with phenylephrine. Both doxycycline and GM6001 significantly reduced the loss in vascular tone mediated by LPS as analyzed by one way ANOVA at the 6 hr timepoint (65 ± 3% of original tone, doxycycline; 65 ± 6% of original tone, GM6001, Figure 3.2B and C). Two-way repeated measures ANOVA analysis reveal differences (*$p < 0.05$) between Control and LPS treated groups at 4 and 6 hr. In the presence of inhibitors, GM6001 and doxycycline significantly prevented the LPS-induced loss in vascular tone which was significant (*$p < 0.05$) at the 4 hr (LPS + Doxy) and 6 hr (LPS + Doxy, LPS + GM6001) groups. Interestingly, LPS-treated groups with MMP inhibitors were significantly different (*$p < 0.05$) than control at the 4 hr (LPS + GM6001) and 6 hr (LPS + Doxy, LPS + GM6001) time points.

MMP inhibition protects IL-1β-mediated vascular hyporeactivity

In the above experiments inhibition of MMPs may have protected against the LPS-mediated dysfunction by inhibiting the proteolytic processing of the IL-1β precursor which could be activated in such conditions [81]. In order to determine whether MMP inhibition could protect against vascular dysfunction downstream of its possible effects on cytokine processing we incubated aortae with IL-1β (10 ng/mL). In the first set of experiments aortae from normal rats were mounted in organ baths and contracted with phenylephrine. At this time point IL-1β (10 ng/mL) was added to the preparations and tone was then monitored for 6 hr. IL-1β at this concentration produced a greater spontaneous loss of tone compared to LPS treated vessels seen in Figure 3.3 (vessels relaxed to 16.4 ± 0.2 % of original phenylephrine-induced tone, Figure 3.4A). Doxycycline significantly inhibited this IL-1β-induced loss of tone in a concentration-dependent manner.

The effect of GM6001 on IL-1β induced hyporeactivity was tested in a separate group of experiments which also facilitated measurement of MMP activity. Fresh aortae were incubated at 37 °C with IL-1β (10 ng/mL) under sterile conditions in culture medium for 6 hr. IL-1β treatment significantly increased MMP-2 activity in the aorta incubation media compared to vehicle treated rings ($^*p < 0.05$, Figure 3.4B). IL-1β decreased the contractile response to increasing concentrations of phenylephrine, relative to control vessels that were incubated without the cytokine ($^*p < 0.05$, Figure 3.4C). The addition of 10 or 30 μM GM6001 to the cell culture medium significantly protected the vessels from cytokine mediated dysfunction in a concentration dependent manner ($^*p < 0.05$, Figure 3.4C). GM6001 had no effect on the contractile response of control vessels, nor did the drug vehicle have any effect on IL-1β treated vessels (data not shown). The maximum response to KCl (75 mM), a vasoconstrictor that acts by electromechanical coupling and not via plasmalemmal membrane receptors, was also significantly decreased with IL-1β treatment ($^*p < 0.05$, Figure 3.4D). The addition of 10 μM or 30 μM GM6001 restored vascular reactivity to KCl to levels not significantly different from control vessels.

LPS administration *in vivo* causes overt signs of endotoxemia

Overt symptoms of endotoxemia were apparent in rats 6 hr following LPS administration. These included lethargic behavior, piloerection, and porphyrin secretion from the eyes. Plasma nitrate/nitrite, measured as a marker of NO biosynthesis, was significantly elevated at this time point relative to vehicle-treated (control) rats (330 ± 37 μM vs. 32 ± 4 μM, respectively, $n = 10$ per group, $p < 0.05$).

Aortic gelatinolytic activity is increased following LPS administration *in vivo*

6 hr after LPS administration, net gelatinolytic activity in excised aortae was significantly increased relative to aortae from control rats (*$p < 0.05$, Figure 3.5A). Since gelatin is susceptible to cleavage by collagenases (MMP-1, -8, -13), collagenolytic activity was also measured. Net collagenolytic activity in the aortae, however, was not increased relative to control rats (Figure 3.5B).

Zymographic analysis of control aortic tissue (Figure 3.6A) revealed robust 72 kDa MMP-2 activity, as well as minor 75 kDa and 64 kDa MMP-2 activities. The rank order of MMP-2 activities was 72 kDa > 75 kDa > 64 kDa. The 72 kDa and 64 kDa bands corresponded to MMP-2 by comparison to the standard, and the 75 kDa band corresponded to a rodent-specific glycosylated form of pro-MMP-2 [244]. The 72 kDa MMP-2 activity was not significantly changed following LPS treatment (Figure 3.6A). In contrast, however, 72 kDa MMP-2 protein content was significantly decreased following LPS (*$p < 0.05$, Figure 3.6B).

In aortae from control rats, gelatinolytic activity of molecular weight higher than 75 kDa (the region for MMP-9) was not detectable. However, when zymographic gels were incubated for a longer period of time, 92 kDa MMP-9 activity could be detected in aortae from LPS treated rats only (*$p < 0.05$, Figure 3.6B). Interestingly, immunoblot analysis revealed that 92 kDa MMP-9 protein content was not significantly different between control and LPS treated rats (Figure 3.6B). As a negative control for these immunoblots, no bands were detected when blots were probed with appropriate non-immune IgG.

LPS administration *in vivo* affects TIMP-1 and TIMP-4 protein content

Immunoblot analysis was performed to assess aortic TIMP-1, -2, -3, and -4 content. TIMP-1 protein was found to be increased almost threefold in aorta from LPS treated rats relative to control rats (*$p < 0.05$, Figure 3.7A). In contrast, TIMP-4 was significantly decreased (*$p < 0.05$, Figure 3.7C). Both glycosylated and non-glycosylated forms of TIMP-2 could be detected, however, they were unchanged in aortae from LPS-treated rats (Figure 3.7B). TIMP-3 could not be detected (data not shown).

Inhibition of MMPs *ex vivo* protects against *in vivo* LPS-induced hyporeactivity

We investigated the ability of MMP inhibition to acutely reverse vascular hyporeactivity following *in vivo* LPS administration. Two aortic rings were mounted in organ baths from each LPS-treated or control rat. Half of the rings were incubated *ex vivo* with doxycycline (100 μM, 10 min), and the other half were treated with ddH_2O vehicle. Vessels from LPS-treated rats were significantly hypocontractile compared to vessels from control animals (Figure 3.8). Doxycycline significantly improved contractile responses to phenylephrine (*$p < 0.05$). *Ex vivo* doxycycline was able to significantly improve the diminished contractile response to KCl in aortae isolated from LPS-treated animals (data not shown). Doxycycline did not significantly change the contractile response of vessels from control animals to either phenylephrine (Figure 3.8) or KCl (data not shown).

3.4 Discussion

We studied the effect of MMP inhibition on LPS and IL-1β-mediated vascular dysfunction, as well as the regulation of vascular MMP and TIMPs during endotoxemia, an *in vivo* model of acute inflammation. Both the LPS-mediated spontaneous loss of contractile tone and IL-1β-mediated vascular hyporeactivity were ameliorated by inhibition of MMP activity. During endotoxemia in rats, both MMP-2 and MMP-9 were detected while net gelatinolytic activity in the aorta was increased. Aortic TIMP-1 was increased, TIMP-4 was decreased, and TIMP-2 remained unchanged in aortae taken from LPS treated rats. This is the first study to demonstrate that MMP inhibition ameliorates inflammatory vascular dysfunction, and that vascular MMPs and TIMPs are acutely regulated *in vivo* by severe inflammatory stress.

Ambient levels of LPS, under standard laboratory conditions, were sufficient to cause a spontaneous and slowly developing relaxation in phenylephrine-contracted rat aortae incubated for up to 6 hr in organ baths. The ability of polymyxin B, a known chelator of LPS [242], to prevent this hyporeactivity suggests that the loss of tone was mediated by ambient LPS under our experimental conditions [108]. The MMP inhibitors doxycycline and GM6001 prevented this loss of tone. In a parallel set of experiments where LPS was controlled, we observed that doxycycline and GM6001 also prevents this decline in tension. Doxycycline is a tetracycline class antibiotic which exhibits MMP inhibitory activity independent of its antibacterial effects [171]. The chemically distinct MMP inhibitor GM6001 (Figure 3.1), which is devoid of antibacterial action, also prevented the LPS-mediated spontaneous loss of contractile tone. Interestingly, previous work demonstrated that this hypocontractility is related to enhanced inducible NO

synthase activity [108,245]. Enhanced NO production under inflammatory conditions leads to increased peroxynitrite formation [72] and cytokine production in the aortic wall, both of which are known to increase MMP activity and decrease TIMPs [157,237,238,246]. Thus, it is possible that MMPs are downstream mediators of the well- characterized increase in endogenous oxidative stress in this model.

Incubating blood vessels *ex vivo* with IL-1β is another well-established model of vascular hyporeactivity to vasoconstrictor agonists [77]. This model was used to test whether MMP inhibitors were protective independent of their ability to inhibit proteolytic cleavage of cytokine precursors. This is particularly important since MMP-2 is known to cleave and activate the IL-1β precursor [81] . IL-1β is thought to be a principal player in the cardiovascular dysfunction associated with endotoxemia since its administration causes marked hypotension in rabbits [26]. Moreover, IL-1β receptor antagonists reduce cardiovascular dysfunction and mortality in models of sepsis [28,30]. In the present investigation treatment with IL-1β stimulated an increase in MMP-2 activity from the isolated vessels, and MMP inhibition with GM6001 significantly improved the contractile response to both phenylephrine and KCl in treated vessels. This suggests that part of the IL-1β mediated response is dependent on MMP activity. As well, GM6001's ability to improve vascular reactivity to both phenylephrine and KCl suggests that it acts at a point downstream of where both receptor- and nonreceptor-dependent contractile pathways converge.

A well-established model of endotoxemia was used to assess whether MMP and TIMPs are regulated acutely *in vivo* during severe inflammatory stress. Previously, we demonstrated that significant cardiovascular dysfunction occurs 6 hr post LPS

administration in the rat. This dysfunction is characterized by hypotension and severe depression of cardiac mechanical function [66,73,75]. Here we demonstrated that severe vascular hyporeactivity also occurs 6 hr post LPS administration. At this time point nitric oxide, superoxide anion, markers of circulating and cardiac peroxynitrite production, and proinflammatory cytokines are all significantly increased [66,72]. Alterations of *in vivo* MMP activity were anticipated since MMPs and TIMPs are regulated by proinflammatory cytokines and peroxynitrite. Net gelatinolytic activity increased in aortae taken from LPS-treated animals. MMP-2 and -9 are largely responsible for gelatinolytic activity, however, the collagenases (MMP-1, -8, and -13) are also recognized to cleave gelatin *in vitro*. In order to determine if collagenases were contributing to the increase in net gelatinolytic activity we also measured collagenolytic activity. The lack of increase in collagenolytic activity suggested that the measurable gelatinolytic activity was due to MMP-2 and -9 and not collagenases.

Increased aortic MMP-9 activity following LPS administration adds to mounting evidence implicating this MMP in *in vivo* models of endotoxemia and septic shock. We previously demonstrated that circulating 92 kDa MMP-9 activity correlated inversely with mean arterial blood pressure in endotoxemic rats [75]. As well, *in vivo* administration of MMP inhibitors (doxycycline or Ro 31-9790) significantly decreased 92 kDa MMP-9 activity and improved cardiac mechanical dysfunction in the hearts from endotoxemic rats [73]. Opdenakker and colleagues have demonstrated that both MMP inhibition and MMP-9 gene deletion significantly protect mice against lethal doses of LPS [196,247]. A significant increase in circulating MMP-9 activity was also noted in human volunteers administered LPS [194] . Finally, in septic shock patients, plasma

MMP-9 protein correlated with circulating LPS levels concentration and was significantly higher in non-survivors than survivors [240].

A dysregulation of aortic TIMPs was also seen following LPS administration *in vivo*. Interestingly, TIMP-4 content was significantly decreased. These observations are similar to the acute loss of TIMP-4 seen in isolated perfused rat hearts following proinflammatory cytokine mediated cardiac dysfunction [200] or in acute ischemia-reperfusion injury [169]. The increase in aortic TIMP-1 was not unexpected since it is regarded as an inducible TIMP [164]. However, it should be noted that the ability of TIMP-1 to inhibit MMP activity may be diminished under conditions of enhanced oxidative stress characterized by increased peroxynitrite biosynthesis [238] . Thus, even though TIMP-1 protein content was elevated, its net inhibitory effect may not have likewise increased.

The apparent discordance between protein levels (as measured by immunoblot) and activity (as measured by zymography) of both 72 kDa MMP-2 and 92 kDa MMP-9 was an interesting finding. This discordance might be attributable to peroxynitrite-induced activation of these enzymes [237]. Peroxynitrite disrupts the MMP propeptide domain 'cysteine switch' by S-glutathiolation of a critical cysteine residue in this domain, causing a conformational change resulting in an activated 'proenzyme' [157,237]. Thus, increased aortic peroxynitrite biosynthesis during endotoxemia [72] may have activated both the 72 kDa MMP-2 and the 92 kDa MMP-9 without loss of the propeptide. With such activation, an increase in MMP-9 activity could be detected in zymography despite unchanged protein content. Likewise, such activation could allow MMP-2 activity to appear equal between LPS and control aortae, despite a loss in MMP-2 protein content in

the LPS aortae. Other explanations also exist for the observed discordance between zymography and immunoblot, such as possible epitope modification by peroxynitrite or protease activity.

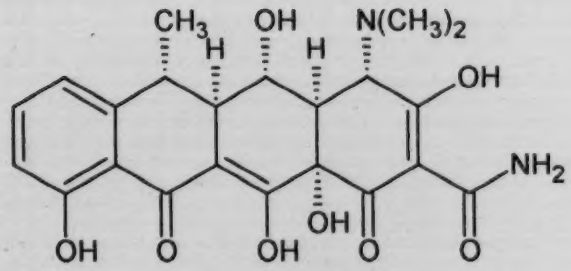
Our study also addressed the functional significance of vascular hyporeactivity following *in vivo* LPS-mediated MMP-TIMP dysregulation. 6 hr after LPS administration to the rat we found that *ex vivo* vascular reactivity was significantly impaired. Doxycycline was able to acutely improve the vascular hyporeactivity to phenylephrine. This raises the possibility that MMP inhibition may be beneficial under *in vivo* conditions of inflammatory stress (e.g. sepsis) when blood pressure has fallen due to the vascular hyporeactivity.

Overall, our results raise several interesting new questions. For instance, the specific target(s) of MMPs in these models of vascular hyporeactivity is unknown. A recent investigation by Chew *et al* suggests that MMPs may interfere with Ca^{2+} entry required for KCl-mediated contractions in the rat aorta [224]. The addition of MMP-2 or MMP-9 to aortic strips isolated from normal rats blunted responses to phenylephrine and KCl, and at the same time interfered with entry of radiolabelled extracellular Ca^{2+}. Whether this mechanism contributes to inflammation-associated hyporeactivity to vasoconstrictors remains to be determined. Some possible limitations to these experiments should be considered. First is the use of micromolar amounts of GM6001, a concentration higher than the reported K_i of this drug when tested against isolated MMP-2 and MMP-9 proteins under cell-free conditions. Such micromolar concentrations are necessary to produce biological effects in intact cells and tissues [189,248]. Thus it is likely that higher concentrations of MMP inhibitors are required to produce their effects

in a complex biological milieu. It should also be noted that, in cell culture models, IL-1β is recognized to stimulate a variety of MMPs in vascular smooth muscle cells [159,159,249] and endothelial cells [250]. Thus, part of the protective effect of GM6001 could be explained by an inhibition of a variety of MMPs.

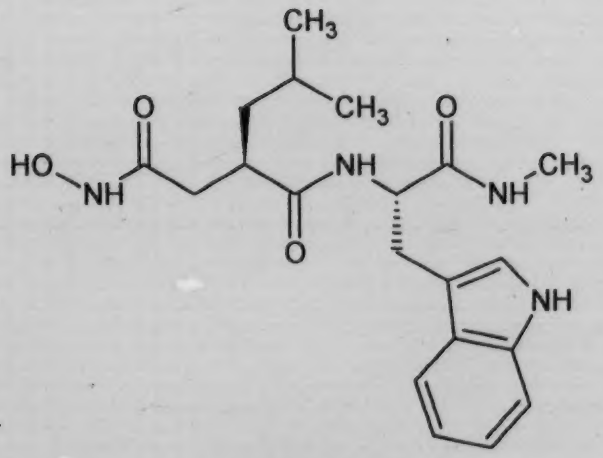
In summary, MMPs and TIMPs are acutely regulated in the vasculature during LPS or IL-1β mediated vascular hyporeactivity. MMP inhibitors are effective in preventing this vascular dysfunction. *In vivo* acute endotoxemia produces vascular dysfunction that is associated with an imbalance between MMPs and TIMPs. This vascular dysfunction was significantly ameliorated after incubation with an inhibitor of MMPs. These data suggest that MMPs play a role in acute inflammatory vascular dysfunction associated with conditions such as endotoxemia.

A



Doxycycline

B



GM6001

Figure 3.1 Structure of MMP inhibitors (A) doxycycline and (B) GM6001

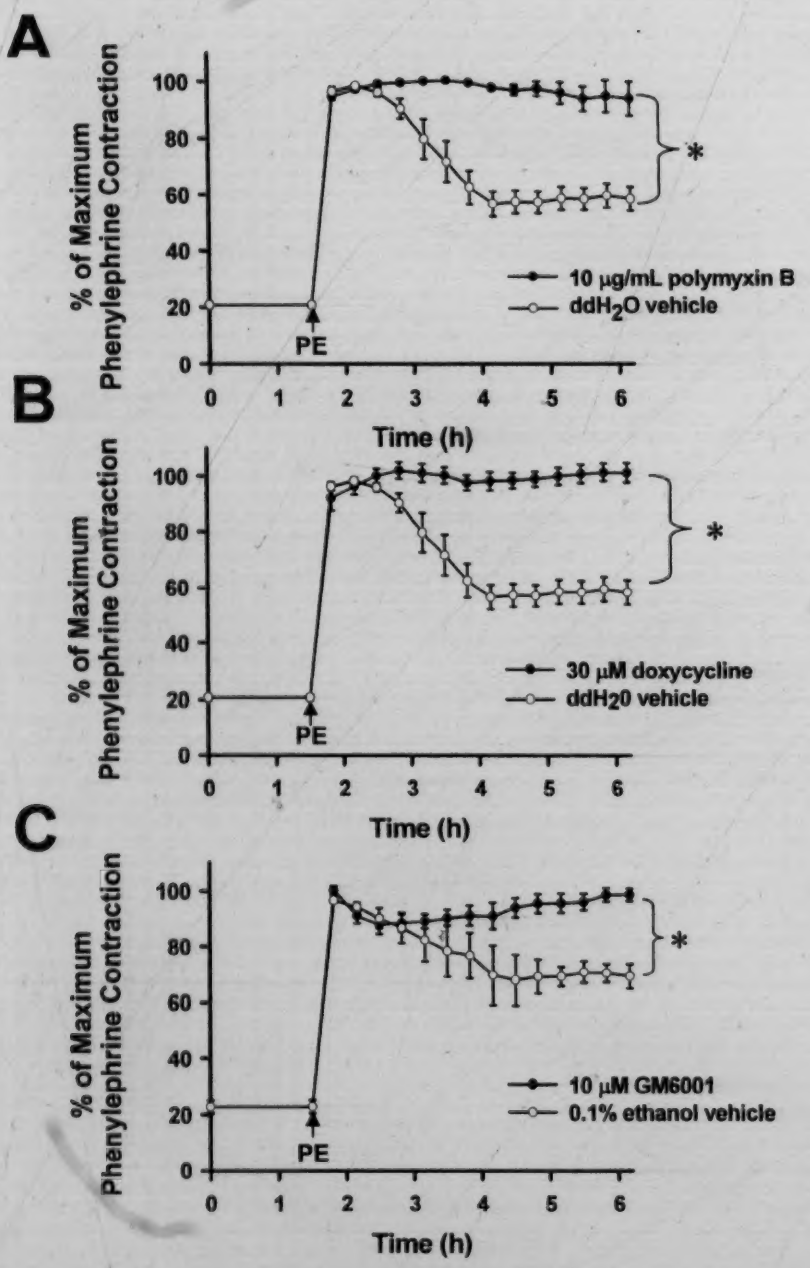


Figure 3.2 Response of aortic rings taken from normal rats to a time-dependent loss of phenylephrine (PE) induced tone. PE was added as indicated by the arrow after 1.5 hr equilibration and then rings were treated with either (A) polymyxin B (10 μg/mL) (B) doxycycline (30 μM) or ddH_2O vehicle or (C) 10 μM GM6001 or 0.1% ethanol vehicle (*$p < 0.05$, two way repeated measures ANOVA, n = 4-5 aortic rings taken from distinct animals/group). Experiments performed by M.M. Lalu and R. Chowdhury.

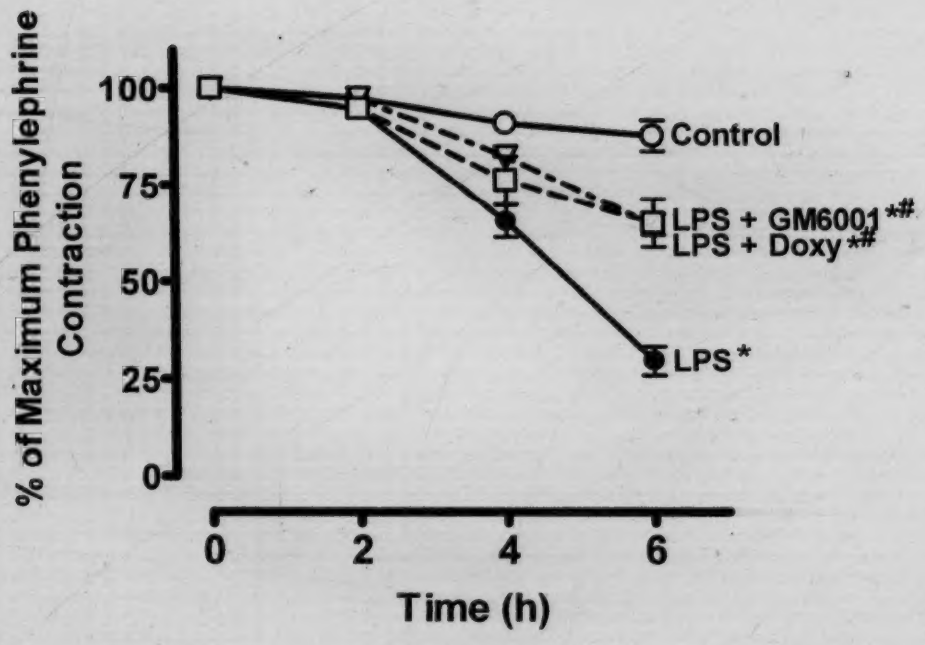


Figure 3.3 LPS-induced time-dependent loss of phenylephrine (PE) induced tone in aortic rings taken from normal rats. After equilibration, LPS was added immediately after PE in the presence or absence of doxycycline (30 μM) or 30 μM GM6001 (*$p < 0.05$ vs control, #$p < 0.05$ vs LPS, two way repeated measures ANOVA, n = 3-5 aortic rings taken from distinct animals/group). Experiments performed by J. Cena.

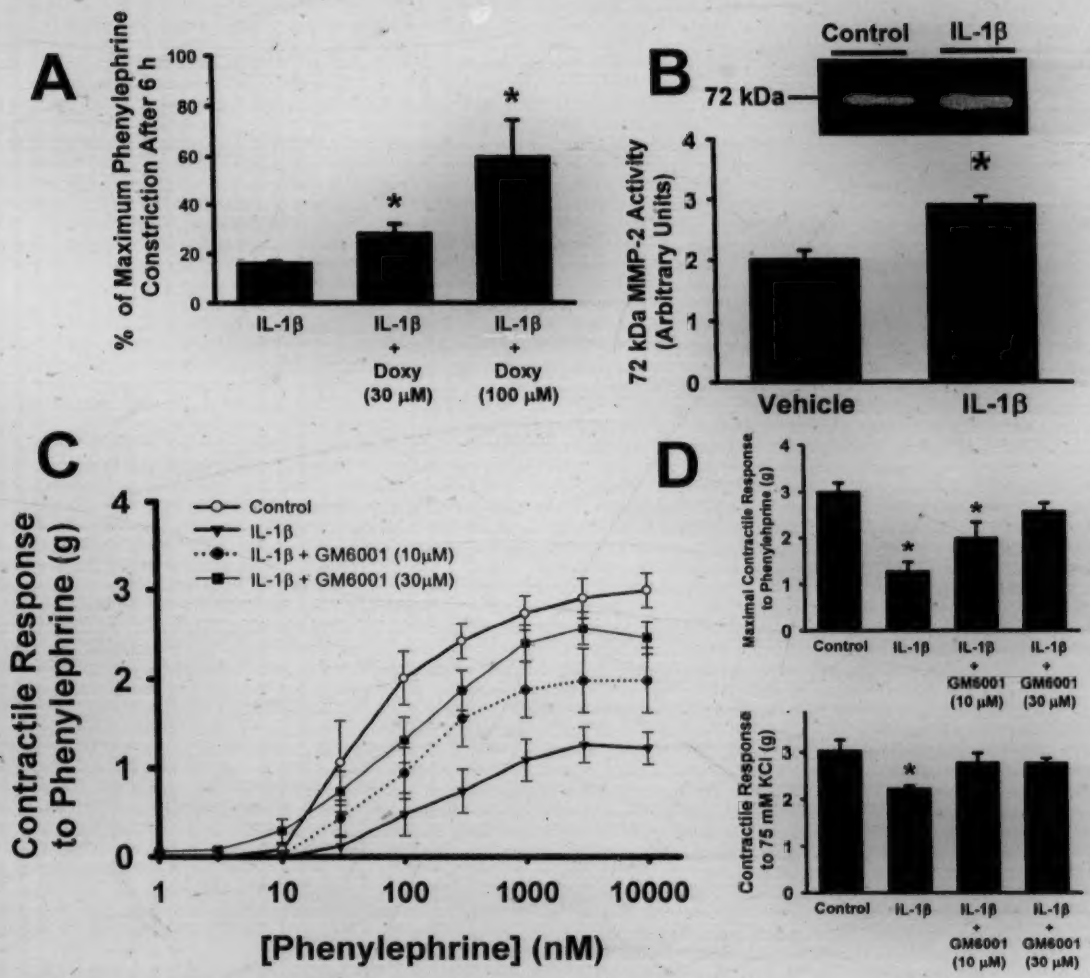


Figure 3.4 (A) Response of aortic rings taken from normal rats to an IL-1β (10 ng/mL)-induced loss of phenylephrine (PE) induced tone. Rings were treated with either doxycycline (30 or 100 μM) or ddH_2O vehicle (*$p < 0.05$ vs IL-1β, one-way ANOVA, n = 3 rings taken from distinct animals/group). (B) A representative zymogram of MMP-2 activity in incubation media of aortic rings taken from normal rats and incubated for 6 hr at 37 °C in the presence or absence of IL-1β (10 ng/mL) (*$p < 0.05$, independent samples t-test, n = 6 rings taken from distinct animals/group). (C) Left: Contractile response of aortic rings taken from normal rats and incubated for 6 hr at 37 °C in the presence or absence of IL-1β (10 ng/mL) ± GM6001 (10 or 30 μM) and then placed in organ baths and exposed to increasing concentrations of phenylephrine. Right: Summary data of maximal contractile response to phenylephrine (*$p < 0.05$ vs Control, one-way ANOVA followed by Fisher's LSD test, n = 8-13 aortic rings taken from distinct animals/group). Right: Contractile response of aortic rings as prepared in (B) to 75 mM KCl (*$p < 0.05$ vs Control, one-way ANOVA followed by Fisher's LSD test, n = 8-13 aortic rings taken from distinct animals/group). Experiments performed by M.M. Lalu, J. Cena, and R. Chowdhury.

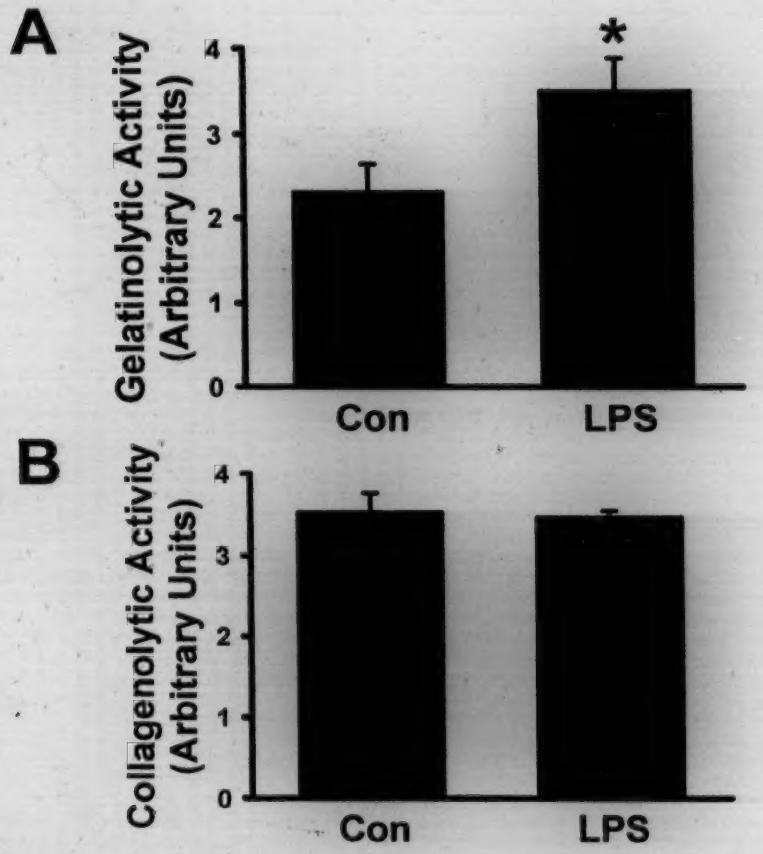


Figure 3.5 (A) Gelatinolytic and (B) collagenolytic activities of homogenates prepared from aorta removed 6 hr after i.p. injection of either lipopolysaccharide (LPS, 4 mg/kg) or pyrogen free water vehicle (Con) (*$p < 0.05$, independent samples t-test, $n = 5$ rats/group). Experiments performed by M.M. Lalu and R. Chowdhury.

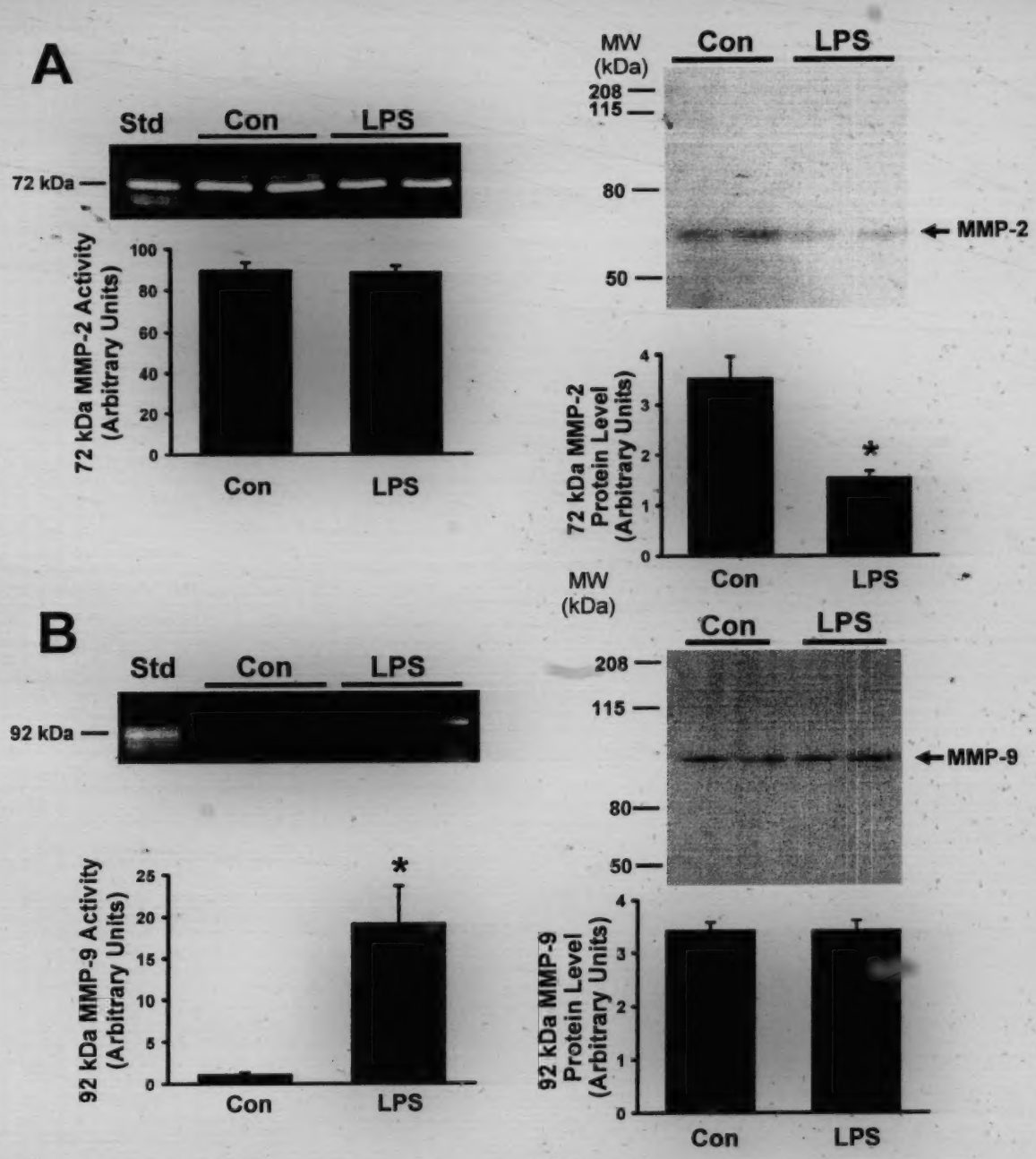


Figure 3.6 (A) MMP-2. Left: A representative zymogram of vascular homogenate MMP-2 activities. Aortae from two control rats (Con) and two lipopolysaccharide treated (6 hr) rats (LPS) show primarily 72 kDa activity. "Std" represents culture media from HT-1080 cells. Gel incubation time: 20 hr. Right: a representative immunoblot showing 72 kDa MMP-2 protein content. Position of molecular weight markers is shown on the left of the immunoblot. (B) MMP-9. Left: A representative zymogram of vascular homogenate MMP-9 activity. 92 kDa activity appears in aortae from two LPS treated rats but not from Con rats. Gel incubation time: 30 hr. Right: a representative immunoblot showing 92 kDa MMP-9 protein content (*$p < 0.05$, independent samples t-test, n = 9-13 rats per group). Experiments performed by M.M. Lalu, R. Chowdhury, J. Cena, and A. Lam.

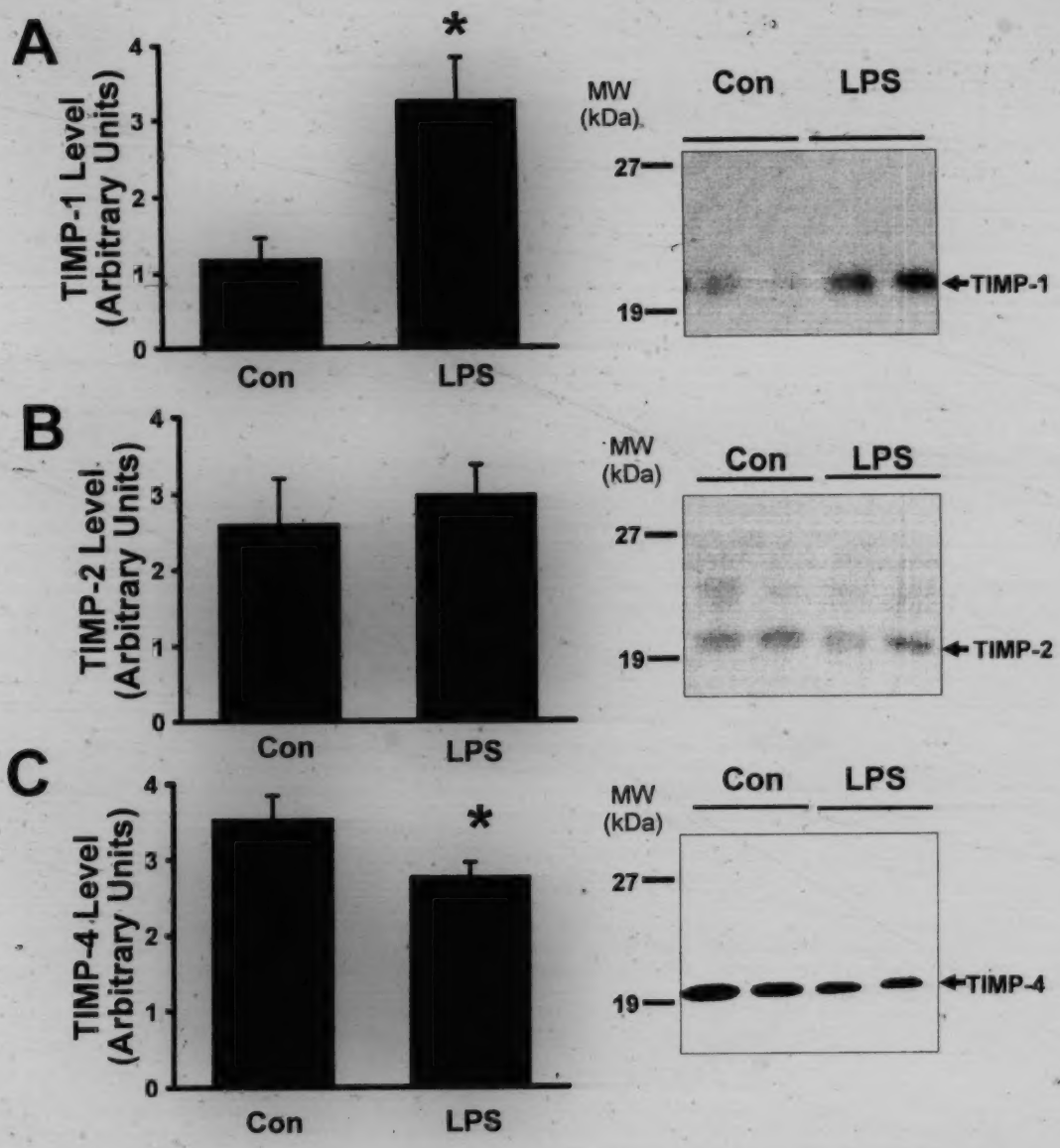


Figure 3.7 (A) TIMP-1, (B) TIMP-2 and (C) TIMP-4 protein content in aortae excised from lipopolysaccharide (LPS, 6 h) or vehicle (Con)-treated rats. TIMP-3 was not detectable (data not shown). Right panels show representative immunoblots taken from aortae from two control rats (Con) and two lipopolysaccharide treated rats (LPS). Position of molecular weight markers is depicted on the left (*$p < 0.05$, independent samples t-test, n = 12-13 rats/group for TIMP-1 and TIMP-4, n = 6 rats/group for TIMP-2). Experiments performed by M.M Lalu, R. Chowdhury, J. Cena, and A. Lam.

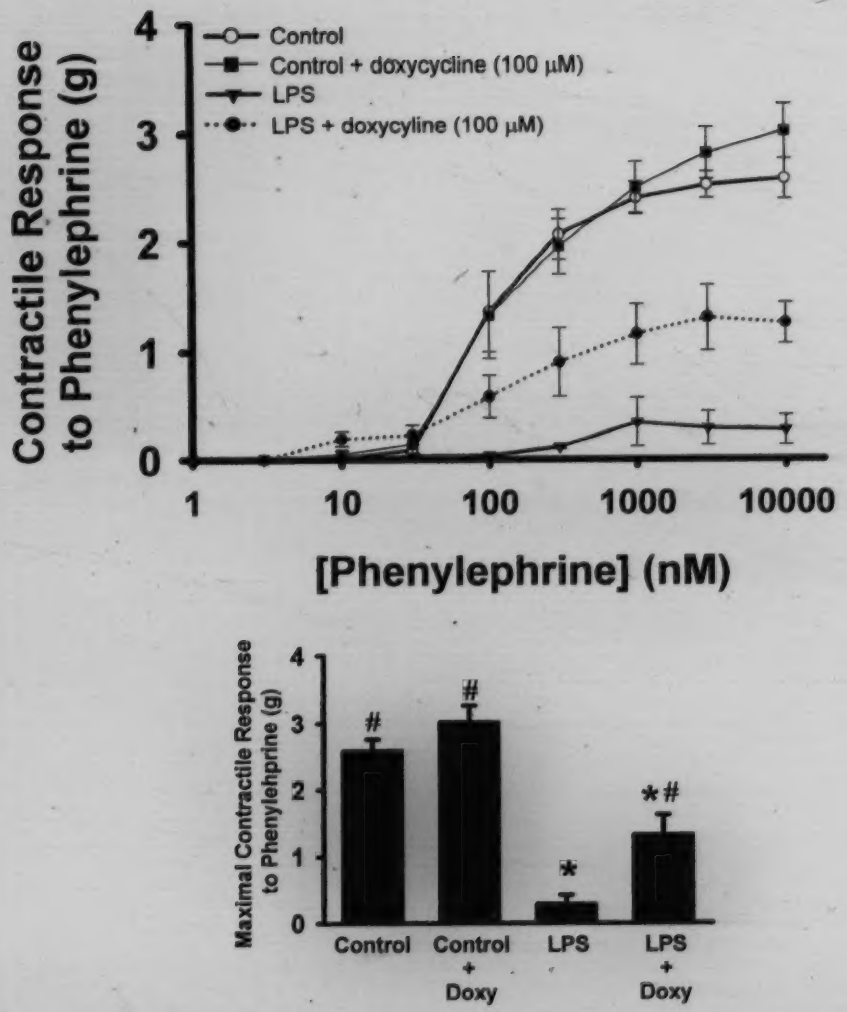


Figure 3.8 Top: Contractile response of aortic rings taken from rats 6 hr after i.p. injection of either lipopolysaccharide (LPS, 4 mg/kg) or pyrogen free water vehicle (Con). Rings were treated *ex vivo* with either doxycycline (100 μM) or ddH_2O vehicle. Bottom: Summary data of maximal contractile response to phenylephrine (*$p < 0.05$ vs Control, #$p < 0.05$ vs LPS, one way ANOVA followed by Fisher's LSD test, n = 3-4 aortic rings/group). Experiments performed by Cena J, and Lalu MM.

CHAPTER 4
ENDOTHELIAL DEPENDENCE OF MATRIX METALLOPROTEINASE-MEDIATED VASCULAR HYPOREACTIVITY CAUSED BY LIPOPOLYSACCHARIDE

A portion of this chapter is published. Cena, J., Lalu, M.M., Rosenfelt, R., Schulz, R. Endothelial dependence of matrix metalloproteinase-mediated vascular hyporeactivity caused by lipopolysaccharide. *Eur J Pharmacol*. 582:116-122, 2008.

4.1 Introduction

Sepsis, an often fatal condition arising from the body's exaggerated response to an infection, is the leading cause of death in intensive care units [1]. Research into the pathogenesis of this condition has revealed many potential targets, however, no effective pharmacotherapy has been discovered. Unravelling the underlying mechanisms is difficult because the natural immunological response to bacterial infection releases a cascade of inflammatory mediators, thus complicating the pathophysiology of sepsis. Initiation of the septic cascade begins with exposure to immunogens in the bloodstream. Lipopolysaccharide (LPS, also known as endotoxin), a normal component of the cell wall in gram-negative bacteria, triggers the immune response.

Cardiovascular symptoms dominate the clinical presentation of sepsis. These are characterized as myocardial dysfunction as well as severe hypotension caused in part by hyporeactivity of the blood vessels to vasoconstrictor agonists [35]. This hyporeactivity poses a severe problem for treating sepsis in that high doses of vasopressor agonists are required to maintain an even less than adequate blood pressure. This phenomenon is due to the insensitivity of the vasculature to these agents [38]. Despite several suggestions, the mechanisms involved in the development of vascular hyporeactivity still remain unclear. Following LPS administration or exposure to proinflammatory cytokines, nitric oxide (NO) production is enhanced via induction of inducible nitric oxide synthase (iNOS) in the endothelium [106], cardiac myocytes [65,107], vascular smooth muscle [110], and neutrophils [109]. iNOS continuously produces high amounts of NO sustained over several hours. Evidence of enhanced NO production is present in septic patients as elevated NO metabolites (nitrite and nitrate) are present in plasma [113,114].

MMPs are a family of zinc-dependent endopeptidases involved in a variety of physiological and pathophysiological processes. MMPs are synthesized as inactive zymogens and can be activated either by proteolytic removal of the pro-peptide domain to reveal the catalytic site or by peroxynitrite mediated oxidation of a critical cysteine sulfhydryl in the pro-peptide domain [163,237]. MMP-2 and -9 (also known as gelatinase A and B, respectively), are involved in the pathophysiology of various cardiovascular diseases [162,251]. MMP-2 is constitutively active and is ubiquitously expressed in nearly all cells throughout the body, whereas MMP-9 is a cytokine-inducible enzyme particularly found in activated neutrophils and macrophages.

The vascular endothelium is involved in several important homeostatic functions which include the regulation of local blood flow and fluid migration through the vascular intima [252]. In the context of sepsis, both endothelial dysfunction [253] and apoptosis [254] contribute to the pathophysiology of the vascular defect. Little is known about the role of MMPs in the dysregulation of the endothelium in the context of septic shock.

Our laboratory demonstrated that inhibition of MMPs is beneficial in both LPS- and interleukin-1β-induced vascular hyporeactivity [74] and myocardial dysfunction [73]. However, the exact mechanisms of MMP action in LPS-induced vascular dysfunction are unclear and to what extent the endothelium is involved is also unknown. It is known that the endothelium accelerates LPS-induced vascular hyporeactivity [255-257] and MMP-2 activity is enhanced in cultured human umbilical vein endothelial cells following LPS treatment [239]. Therefore we examined the dependence of the endothelium in the involvement of MMPs in LPS-induced vascular hyporeactivity *in vitro*.

4.2 Materials and Methods

Animals

Animals are described in Section 2.1.

LPS-induced vascular hyporeactivity

The protocol is described in Section 2.3.

Endothelial denudation

After aortae were cleaned of connective tissue, a corrugated paperclip was inserted into the lumen of the aorta and the endothelium was removed by light mechanical abrasion. Following equilibration rings were contracted with phenylephrine (750 nM) and at the plateau of contraction, acetylcholine chloride (10 μM) was added to assess the integrity of the endothelium. Endothelium-denuded rings which relaxed by greater than 15% of phenylephrine-induced tone were not used.

Preparation of aortae homogenates

This protocol is described in Section 2.7.

Measurement of MMP activity by zymography

This protocol is described in Section 2.9.

Statistical Analysis

This is described in Section 2.11.

4.3 Results

Endothelial-dependence of NOS and MMP inhibition in LPS-induced vascular dysfunction *in vitro*

To ensure that our model of vascular hyporeactivity was indeed due to an increase in NO biosynthesis caused by induction of iNOS [108], we tested the effects of L-NAME in the presence or absence of intact endothelium. L-NAME prevented LPS-mediated hyporeactivity in both endothelium intact and denuded aortae. In endothelium intact rings, control vessels maintained a contraction of 76 ± 5% (n = 9) of original phenylephrine-induced tone over the 6 hr observation period (Figure 4.1). At the end of the incubation time with LPS, rings relaxed to 33 ± 4% (n = 8) over time which was significantly different at the 4 and 6 hr time points. In the presence of L-NAME rings maintained 80 ± 3% (n = 4) of the original tone which was significantly different from LPS treated rings at the 4 and 6 hr time points. In endothelium-denuded control rings, contraction fell to 80 ± 3% (n = 8) at the end of the incubation period (Figure 4.1B). In the presence of LPS the contraction over time dropped to levels significantly different from control (62 ± 4%, n = 8), whereas in those treated with L-NAME the contraction was maintained over time to 90 ± 3% of original phenylephrine-induced tone (n = 4).

The MMPs inhibitor doxycycline significantly reduced the LPS-induced hyporeactivity over time in endothelium-intact rings to 66 ± 2% (n = 9, Figure 4.1C) at the 4 and 6 hr time points but not in endothelium-denuded rings (71 ± 6%, Figure 4.1D). Doxycycline alone did not cause any changes in phenylephrine-induced tension of either endothelium-intact or denuded rings (n = 3 for each, data not shown).

The protective effect of doxycycline was compared to that of another MMP inhibitor, GM6001, in endothelium-intact rings (Figure 4.2). GM6001 significantly reduced LPS-induced hyporeactivity over time as demonstrated by differences at the 6 hr time point (Control: 88 ± 4%, n = 3; LPS: 30 ± 4%, n = 5; LPS + GM6001: 65 ± 6%, n = 4). Vessels incubated with LPS in the presence of GM6001 were significantly different from controls at the 4 and 6 hr time points.

Zymographic analysis of endothelium intact and denuded aortae

Given that mechanical forces exerted on isolated blood vessels, such as the effects of denuding the endothelium, could possibly affect MMP activity in the vascular wall [258] we first determined the effect of endothelium denudation itself on MMP-2 activity in isolated aortic rings. Identical to previous experiments, these isolated aortae were mounted in the organ bath, contracted with phenylephrine, functionally tested for the presence or absence of endothelium using acetylcholine, washed, and then subjected to a 6 hr phenylephrine-induced contraction before freezing and preparation of tissue extracts. Bands of gelatinolytic activity in the zymograms of the tissue extracts corresponding to MMP-2 were observed at 75, 72, and 64 kDa with relative activities 72 >> 75 >> 64 kDa in accordance with our previous study [74] (Figure 4.3A). In vessels not stimulated with LPS, there were no differences in any of these MMP-2 activities in comparing endothelium intact or denuded aortae. No bands representing MMP-9 activity (92 and 84 kDa) were observed. MMP-2 bands were no longer seen if the zymography incubation buffer contained the MMPs inhibitor doxycycline (30 μM), GM6001 (30 μM) or *o*-phenanthroline (10 μM) (data not shown).

In endothelium-intact aortae, 6 hr LPS treatment resulted in a significant upregulation of 72 kDa MMP-2 activity compared to controls (243 ± 18% of control, n = 3) (Figure 4.3B). This increase was partially inhibited by doxycycline to a level not significantly different from control (166 ± 27%, n = 3). In endothelium-denuded aortae LPS did not significantly change 72 kDa MMP-2 activity (n = 3, Figure 4.3C). There were no significant differences in 75 kDa MMP-2 activity between the treatment groups, either with or without endothelium (n = 3 for each, Figure 4.3D + 4.3E). 64 kDa MMP-2 activity was barely detectable and was not significantly different among groups (data not shown) whereas MMP-9 associated bands were below the detection limit.

4.4 Discussion

This study demonstrates that inhibition of MMPs significantly reduces LPS-induced vascular hyporeactivity. Moreover, this inhibition was observed only in the presence of the endothelium. Functionally this reveals that the endothelial-dependent component of LPS-induced vascular hyporeactivity may be mediated by MMPs. These results also indicate an endothelium-independent component of LPS-induced vascular hyporeactivity. Zymographic analysis showed a significant increase in MMP-2 activity in endothelium-intact aortic rings treated with LPS whereas doxycycline, a tetracycline antibiotic which possesses MMP inhibitory activity independent of its anti-bacterial action [171], decreased MMP-2 activity. These changes were absent in endothelium-denuded rings, further supporting the role of the endothelium in the development of vascular hyporeactivity by MMPs induced by LPS. GM6001, a chemically distinct MMP

inhibitor, also abolished LPS-induced vascular hyporeactivity in endothelium-intact rings. The effect of GM6001 was not tested in endothelium-denuded rings.

Several groups have examined the possible role of MMP-2 and -9 in modulating vascular tone [181,224]. In the former study, MMP-2 and -9 were found to inhibit phenylephrine or KCl-induced Ca^{2+} entry in isolated rat aorta, supporting the notion that MMPs have a vasodilatory role. The latter study using isolated venous vessels demonstrated that MMP-2 may act through opening K^+ channels. There is evidence supporting the activation of K^+ channels in vascular hyporeactivity to catecholamines in septic shock [259] although a clinical study using the K^+ channel inhibitor glibenclamide was equivocal [61]. The relationship between K^+ channels and MMPs in septic shock requires further investigation.

Our laboratory demonstrated the upregulation of MMP activity in both *in vitro* and *in vivo* models of septic shock [74]. In that study, MMP inhibitors were found to partially inhibit the development of hyporeactivity to vasoconstrictors *in vitro* in both cytokine and LPS-treated vessels while an upregulation of MMP-2 and MMP-9 activities was observed in the aortae of rats treated with LPS *in vivo*. In other studies examining the regulation of MMP-2 and -9 *in* vitro, one study showed the activation and release of MMP-2 in response to LPS in cultured endothelial cells [239]. In another study examining murine peritoneal macrophages *in vitro*, LPS was found to cause an increase in MMP-2 and -9 activities [192]. A study analyzing isolated human blood revealed that neutrophils were responsible for the secretion of MMP-9 in response to LPS [193].

In the heart, our laboratory has shown a decrease in MMP-2 activity and protein level in rat hearts 6 hr after LPS injection while no evidence of MMP-9 activity was

found in either normal or LPS-exposed hearts [75]. In the same study, plasma MMP-2 activity and protein levels decreased 6 hr after LPS treatment whereas MMP-9 levels decreased at 12 hr. In another study examining *in vivo* models of sepsis, MMP-9 activity is elevated in the plasma of *Escherichia coli* treated baboons [195]. In humans, MMP-9 plasma concentration and mRNA in peripheral blood monocytes were significantly higher in non-surviving patients with septic shock versus surviving patients [240]. This study also showed a positive correlation between LPS and MMP-9 concentration in the plasma, suggesting a possible role for this enzyme as an indicator of or contributor to septic shock severity.

It is well established that NO has a central role in the pathogenesis of vascular hyporeactivity seen in sepsis [115]. Our results support the role for the pathophysiological generation of NO which contributes to vascular hyporeactivity as the NO synthase inhibitor L-NAME completely prevented LPS-induced vascular hyporeactivity in both endothelium intact and denuded vessels. Together these data support the notion that the mechanisms contributing to LPS-induced vascular hyporeactivity include endothelium-dependent and -independent mechanisms. The endothelium-dependent mechanism is due to both MMPs and NO, whereas the endothelium-independent mechanism is mainly due to NO.

It has been shown that, in aorta isolated from endotoxemic rats, NO rapidly reacts with superoxide to form peroxynitrite [72]. As MMPs are activated by low micromolar concentrations of peroxynitrite [237], we believe that could be the mechanism by which an increase in MMP-2 activity was observed in the aorta. A synergistic action of MMPs and peroxynitrite may occur in the presence of the endothelium whereas only a

NO/peroxynitrite-dependent hyporeactivity occurs in the absence of the endothelium. Our laboratory has recently discovered an inhibitory association of caveolin-1 with MMP-2 [162]. Moreover, caveolin-1 has been shown to colocalize with MMP-2 in endothelial cells [260]. Therefore it would be logical to assume a loss of caveolin-1 (which was shown to occur in LPS-stimulated macrophages [261]) may result in increased MMP-2 activity.

This study has important implications and provides insight into the mechanisms of vascular hyporeactivity. Firstly, not only does this support the involvement of MMPs in LPS-induced vascular hyporeactivity, but establishes the essential role of the endothelium in this mechanism. Kim *et al.* (2000) showed that MMP-2 is activated and released from endothelial cells in response to LPS stimulation [239]; whether this mechanism occurs in our model remains to be examined. *In vivo* models of sepsis using MMP inhibitors show marked protective actions including an improvement in blood pressure [198]. The targets of MMPs in LPS-induced vascular hyporeactivity need to be elucidated. Whether the actions of MMPs on vascular tone are via proteolysis of known susceptible targets either outside (eg. big endothelin, [225]; calcitonin gene-related peptide [262]) or inside the cell (eg. cytoskeleton [263]) remains to be determined.

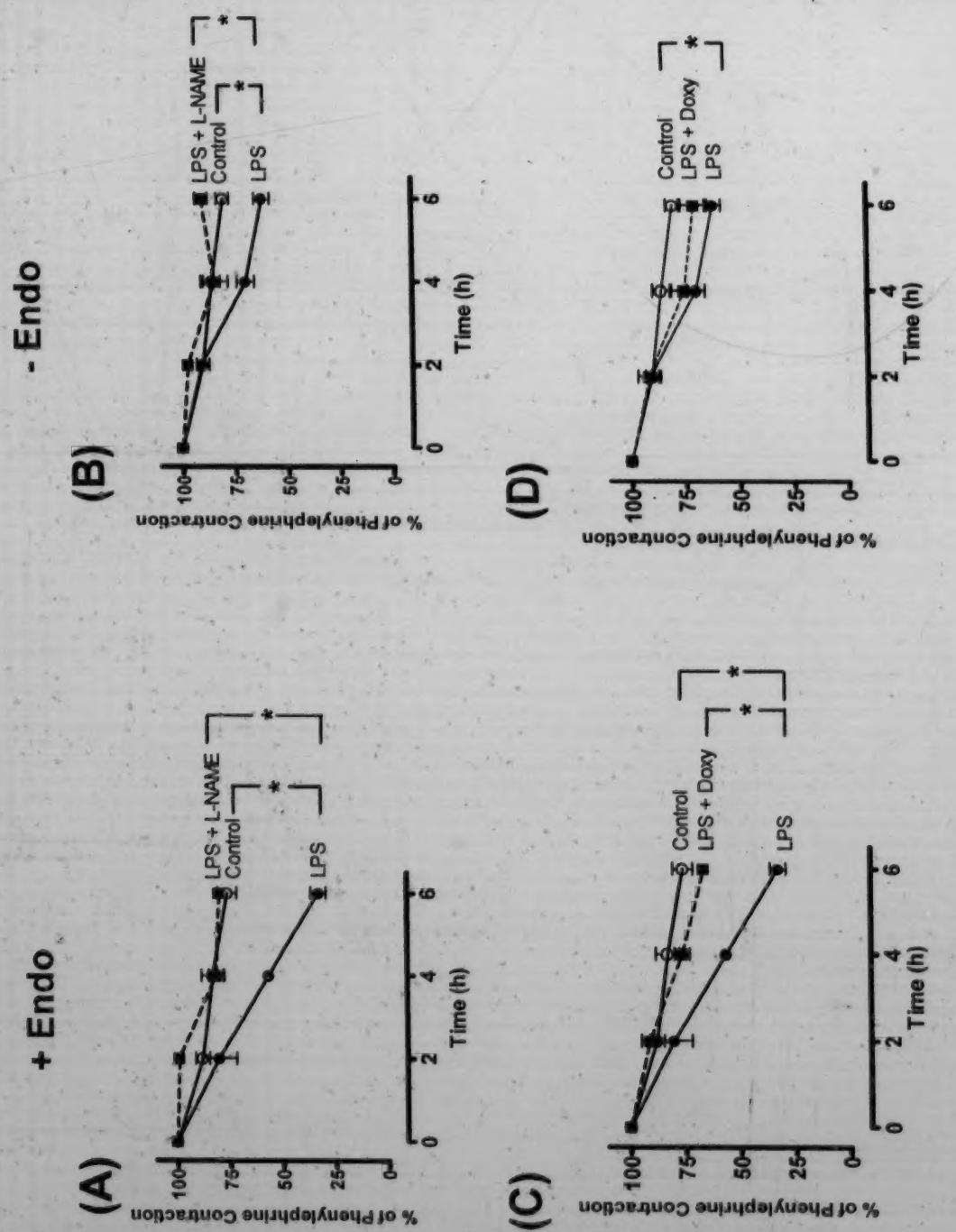


Figure 4.1 NOS and MMP dependence of LPS-induced vascular hyporeactivity. Response of endothelium-intact (A) or endothelium-denuded (B) aortic rings from normal rats to LPS-dependent (300 ng/ml) loss of phenylephrine-induced tone (750 nM). Rings were treated with L-NAME (30 μM) or vehicle (water). Endothelium-intact (C) or endothelium-denuded (D) rings were treated with doxycycline (Doxy, 30 μM) or vehicle (water). Two-way repeated measures ANOVA, n= 5-9 aortic rings/group, *$p < 0.05$.

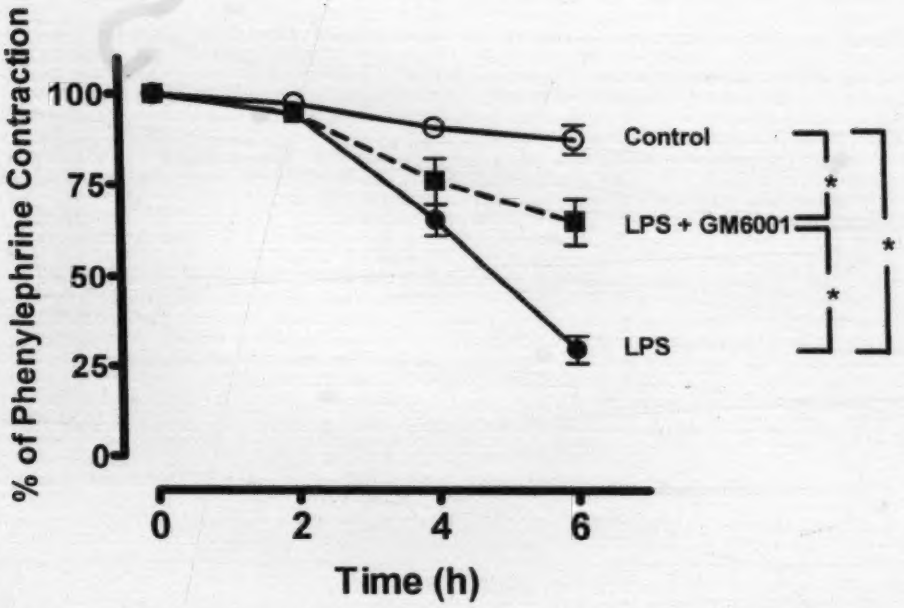


Figure 4.2 The MMP inhibitor GM6001 also reduces LPS-induced vascular hyporeactivity. Response of endothelium-intact aortic rings from normal rats to LPS-dependent (300 ng/mL) loss of phenylephrine-induced tone (750 nM). Rings were treated with GM6001 (30 μM) or vehicle (DMSO). Two way repeated measures ANOVA, n = 3-5 rings/group, *$p < 0.05$.

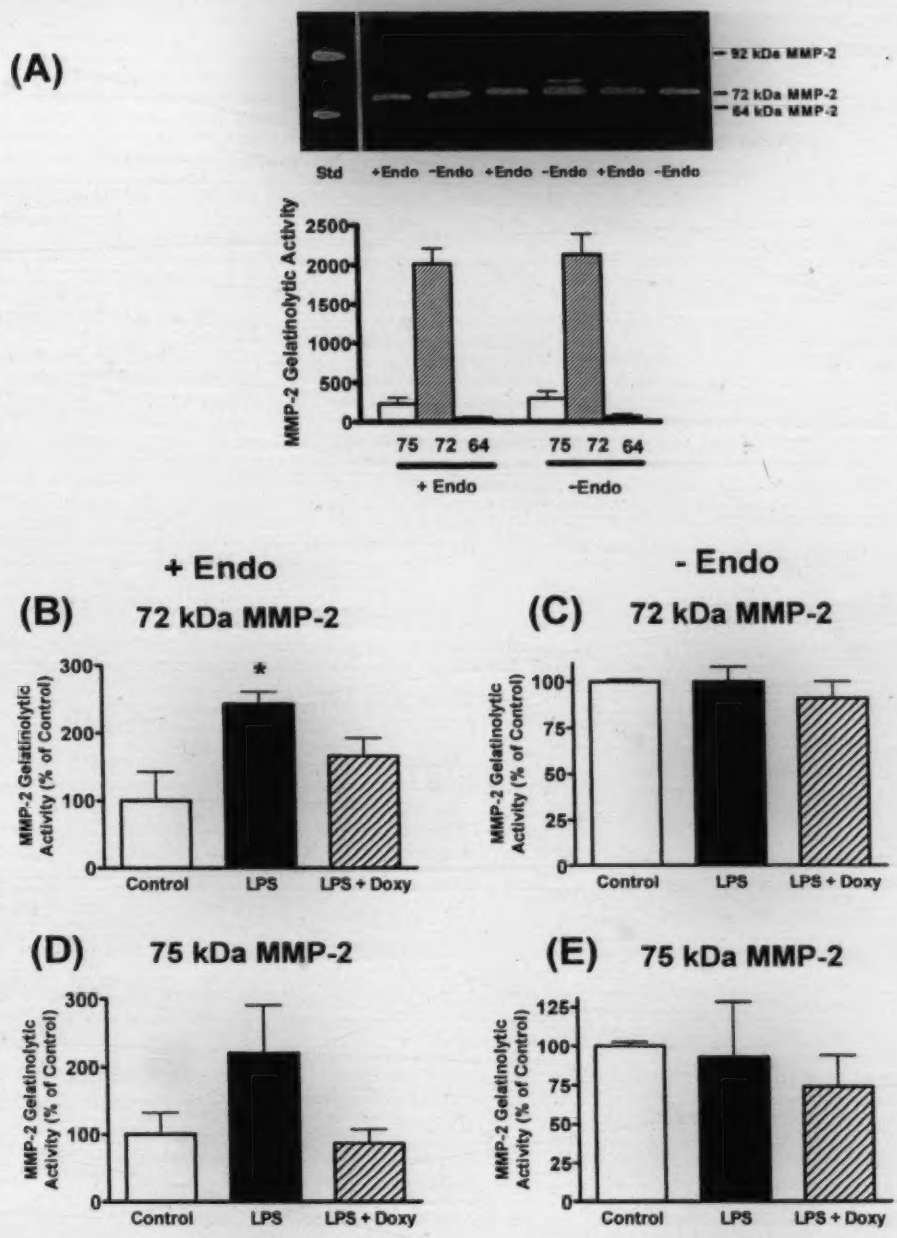


Figure 4.3: (A) Gelatin zymogram (upper panel) showing MMP-2 gelatinolytic activities in endothelium-intact (+Endo) and endothelium-denuded (-Endo) aortic rings and quantification of gelatinolytic activities (lower panel, n = 3 for each). Std: culture medium from HT-1080 cells was used as a standard for MMP-2 and -9 activities. 72 kDa MMP-2 gelatinolytic activity of +Endo (B) or –Endo (C) aortic rings at 6 hr. 75 kDa MMP-2 gelatinolytic activity of +Endo (D) or –Endo (E) aortic rings at 6 hr. Aortae from two different rats were pooled into one experimental sample, n = 3. One-way ANOVA, *$p < 0.05$ vs Control.

CHAPTER 5
INHIBITION OF MATRIX METALLOPROTEINASE ACTIVITY *IN VIVO* PROTECTS AGAINST VASCULAR HYPOREACTIVITY IN ENDOTOXEMIA

A version of this chapter is in preparation: Cena, J., Lalu, M.M., Cho, W.J., Chow, A.K., Bagdan, M., Daniel E.E., Schulz, R. Inhibition of matrix metalloproteinase activity *in vivo* protects against vascular hyporeactivity in endotoxemia.

5.1 Introduction

Sepsis remains the leading cause of death in North American intensive care units [1]. It is characterized by both an infection and a systemic inflammatory response. Since the cardiovascular symptoms of sepsis dominate its clinical presentation, it is important to understand the mechanisms involved in its cardiovascular pathophysiology. Important symptoms include intrinsic myocardial dysfunction and a marked persistent arterial vasodilation. The development of persistent arterial vasodilation involves the emergence of two interacting factors: overproduction of endogenous vasodilatory substances and vascular hyporeactivity to vasoconstrictors [36]. Vasodilators overproduced in septic shock include both nitric oxide (NO) and prostaglandins. Vascular hyporeactivity to vasoconstrictors is characterized by the reduced sensitivity of the vasculature to vasopressor agents. A large body of evidence supports the pathophysiological generation of excess NO in septic shock through the expression of inducible NO synthase (iNOS) in the vasculature which contributes to both vasodilation and vascular hyporeactivity [50,70,72,108,110,264,265].

NO combines with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), a highly reactive species involved in lipid peroxidation, protein and DNA damage, and ion channel and transporter malfunction [266]. Its biosynthesis is enhanced in aortae from LPS-treated rats [72]. $ONOO^-$ activates matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases [237] that are involved in a number of physiological and pathological processes and several cardiovascular pathologies [159,267,268]. $ONOO^-$ directly activates the zymogen form of MMPs without requiring the proteolytic removal of the propeptide domain, the latter form of activation important in the canonical

extracellular actions of MMPs [269]. Since there is increased oxidative stress in the form of $ONOO^-$ in the heart [66,135] and vasculature [270] during sepsis, we hypothesized that MMP activity would also be increased. Previous studies have demonstrated that the mRNAs for several MMPs are upregulated in the liver, spleen, and kidney after bacterial lipopolysaccharide (LPS) administration [271,272], and that selective deletion of the MMP-9 gene protects against LPS-induced mortality [196]. Another investigation demonstrated that broad spectrum MMP inhibition using a chemically-modified tetracycline (without anti-bacterial properties) significantly improved LPS-induced hypotension in pigs [197]. Our laboratory has utilized a model of LPS-induced vascular hyporeactivity in which rats are injected with LPS *in vivo*. *Ex vivo* analysis of aortae from these rats revealed an increase in MMP-2 and -9 activities [74]. Moreover, this study showed the beneficial effects of MMP inhibition in improving vascular hyporeactivity to vasoconstrictors using isolated rat aorta treated with LPS or interleukin-1β *in vitro*. To date, however, no study has examined the effect of MMP inhibition *in vivo* on changes in vascular hyporeactivity following LPS treatment. We therefore tested the hypothesis that MMPs contribute to the development of vascular hyporeactivity to vasoconstrictors using a model of acute endotoxemia in rats and that inhibition of MMPs with doxycycline would protect the loss in vascular contractile tone.

Tetracyclines, a class of antibiotics which have a bacteriostatic effect on microorganisms, have recently been discovered to also inhibit MMP activity [171]. Of the tetracycline class, doxycycline is the most potent MMP inhibitor and exhibits MMP inhibition *in vivo* at plasma levels that are sub-antimicrobial [174]. The use of tetracyclines as MMP inhibitors is widespread particularly in the areas of cardiovascular

[273], cancer [274], and inflammation [171] research. As such, the use of tetracyclines have been examined for their therapeutic potential in models of endotoxic shock [197,198]. Our laboratory has previously shown that *in vivo* doxycycline reduced the depression in cardiac contractile function in LPS-treated rats [73]. As *in vitro* doxycycline protected against LPS-induced vascular hyporeactivity in isolated rat aortae [74], here we examine whether *in vivo* doxycycline prevents vascular hyporeactivity to vasoconstrictors in aorta taken from LPS-injected rats and the associated changes in MMP-2 and -9 in both plasma and the aorta.

5.2 Materials and methods

Animals

Animals are described in Section 2.1.

Rat model of endotoxemia

The protocol for LPS-induced endotoxemia in rats described in Section 2.5.

Tissue preparation and cryosections

Aortae were rapidly excised and connective tissue was trimmed away while in Krebs buffer (118 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7\ H_2O$, 2.5 mM $CaCl_2 \cdot 2\ H_2O$, 11.1 mM D-glucose, 25 mM $NaHCO_3$) bubbled with carbogen (95% O_2-5% CO_2). Aortae were either cut into 5 mm cylindrical segments (two from one aorta) for organ bath studies (see below) or fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.2 - 7.4) for 4 hr at room temperature.

The fixed aortae were rinsed with 0.1 M PB and cryoprotected in 30% sucrose in phosphate buffered saline (PBS) overnight at 4 °C. The cryoprotected aortae were

embedded into embedding molds (Cat. No. 18646A, Polysciences, Inc.) filled with Tissue-Tek® optimal cutting temperature (O.C.T.) compound at -25 °C. 6 μm cryosections were obtained using a cryostat (Leica CM 1850 / CM 1900, Leica Microsystems Inc.) and were attached onto glass slides coated with 1.5% 3-aminopropyltriethoxysilane in acetone. The cryosections were preserved at -80 °C until used.

Assessment of vascular reactivity

Functional analysis is described in Section 2.6.

Preparation of aorta homogenates

Preparation of aortic homogenates for biochemical analysis is described in Section 2.7.

Measurement of MMP activity by zymography

Zymographic analysis is described in Section 2.9.

Western immunoblotting analysis

The protocol for immunoblot analysis is described in Section 2.10., the following primary antibodies were used: an anti-mouse MMP-2 antibody (1:1000 dilution, MAB3308, Chemicon), an anti-mouse MMP-9 antibody (1:1000 dilution, MAB3309, Chemicon), or an anti-mouse iNOS antibody (1:1000, N32020/L20, Transduction Laboratories).

Immunolabeling

The cryosections from -80 °C were dried for 30 min followed by washed with 0.3 % Triton X-100 in PBS twice and then once with PBS alone. The cryosections were

incubated with 10% normal donkey serum (566460, Calbiochem) to reduce artificial staining of non-specific proteins for 1 hr before applying primary antibody. Mouse anti-human MMP-2 IgG_{1kappa} (1:200, MAB3308, Chemicon International), which does not cross react with human MMP-9, and mouse anti-MMP-9 IgG_{1kappa} (1:200, MAB3309, Chemicon International), which does not cross react with human MMP-2, were used as primary antibodies. As a secondary antibody Cy3-conjugated donkey anti-mouse IgG (1:20, 715-165-151, Jackson ImmunoResearch Laboratories) was used. During the incubation with all antibodies, 2% normal donkey serum of total incubation volume was added for stabilization of antibodies. All immunolabeling procedures were performed at room temperature. To determine specificity of immunolabeling, primary or secondary antibody was omitted.

Confocal microscopy

The immunolabeled cryosections were observed by single photon confocal microscope (LSM 510, Carl Zeiss Co.) and saved by LSM 5 Image (Carl Zeiss Co.). Cy3 (red) was scanned by helium / neon laser (543 nm laser line wavelength) with a long pass 560 filter (560 – 700 nm excitation). All images obtained from confocal microscope were enhanced by brightness, contrast, and gamma tool of LSM 5 image and PhotoShop (Version 7.0, Adobe).

Extraction of plasma

Blood samples were obtained from the same rats utilized for functional and biochemical analysis described above. A 17 gauge needle laced with heparin (LEO Pharm Inc.) was used to draw from the chest cavity immediately after sacrifice. The

plasma fraction was obtained following centrifugation (6500 × g for 5 min at 4 °C) and stored at -20 °C for later determination of MMP activities.

Determination of protein content

Analysis of protein content for equal protein loading in western immunoblotting and gelatin zymography is described in Section 2.8.

Statistical analysis

Statistical analysis is described in Section 2.11.

5.3 Results

LPS injection causes overt signs of endotoxemia

LPS but not its vehicle caused overt symptoms of endotoxemia in the rats which were clearly apparent 6 hr after injection. These included lethargic behavior, piloerection, and porphyrin secretion from the eyes. Doxycycline treatment however, did not reduce the development of these symptoms.

Concentration-response to phenylephrine in aorta from endotoxemic rats

LPS injection caused a significant rightward shift in the EC_{50} (Control: pEC_{50} 7.44 ± 0.06; LPS: pEC_{50} 6.73 ± 0.17, n = 7 each; Table 5.1) and a reduction in the E_{max} (LPS: E_{max} 1.46 ± 0.24 g; Control: E_{max} 3.27 ± 0.11 g; n = 7 each, Figure 5.1A and B). Doxycycline administered 30 min after LPS significantly reduced the rightward shift and improved the maximal response in the concentration-response curve to PE (LPS + Doxy: pEC_{50} 6.97 ± 0.10, E_{max} 2.23 ± 0.22 g, n = 7). Aortae from control animals administered doxycycline showed no significant alterations in their response to PE (Control + Doxy: E_{max} 3.20 ± 0.04 g, pEC_{50} 7.21 ± 0.07, n = 3, Figure 5.1B and Table 5.1). LPS also caused

a decrease in response to 75 mM KCl (LPS: 1.65 ± 0.13 g; Control: 2.61 ± 0.15 g, n = 7 each, Figure 5.1C) which was prevented by injection of doxycycline (LPS + Doxy: 2.38 ± 0.10 g, n = 7). Doxycycline administration did not alter contraction to KCl in control rats (Control + Doxy: 2.77 ± 0.26 g, n = 3).

Aortic iNOS protein levels

Some studies have shown a relationship between tetracyclines and their ability to decrease iNOS protein expression via destabilization of iNOS mRNA [275,276]. In this model of endotoxemia, aortic expression of iNOS was induced 6 hr after LPS injection. However, aortic iNOS was not significantly different in LPS treated rats with or without doxycycline (Figure 5.2). This indicates that the protective effects of doxycycline on the development of vascular hyporeactivity *in vivo* are either downstream or independent of actions involving iNOS.

Aortic homogenate protein content and MMP activities

Zymographic analysis of homogenized aortic tissue (Figure 5.3A) revealed robust MMP-2 activity at 72 kDa. 75 kDa bands were below the detection limit and non-quantifiable. LPS did not change aortic net MMP-2 activity significantly from control, nor was there any additional effect of doxycycline treatment. Western immunoblotting analysis of aortic tissue revealed a trend towards a decrease in MMP-2 protein in aortae from rats treated with LPS (Figure 5.3B). Doxycycline also did not significantly alter MMP-2 protein expression after LPS treatment. Analysis of MMP-2 activity expressed per unit protein revealed a significant upregulation of specific MMP-2 activity in aortae from LPS treated rats; doxycycline did not alter net MMP-2 activity (Figure 5.3C).

MMP-9 protein was not altered amongst all experimental groups (data not shown) and its activity was undetectable in the aortic homogenate by zymography.

Immunohistochemistry of aortic cross sections

To preliminarily assess the specificity of the MMP antibodies and the distribution of MMP-2 and -9, we examined the immunohistochemical staining of aorta from control and LPS treated rats using several different MMP-2 and MMP-9 antibodies from different sources. We observed a consistent decrease in MMP-2 but not -9 staining in the aortas of LPS treated rats (data not shown). Among these antibodies, a MMP-2 antibody (MAB3308, Chemicon) and a MMP-9 antibody (MAB3309, Chemicon) were selected and used for further analysis.

Immunohistochemical analysis of aortic cross sections from control aortas revealed ubiquitous MMP-2 distribution throughout the aortic wall in both endothelium and smooth muscle cells (Figure 5.4). 6 hr after LPS administration, a decrease in MMP-2 associated staining was observed in the aortic wall. Doxycycline did not affect the decrease in MMP-2 protein levels; however, a redistribution of MMP-2 towards the intima was apparent. MMP-9 distribution throughout the aortic wall remained unchanged amongst all experimental groups.

Plasma gelatinolytic activity

Plasma from control animals possessed strong MMP-2 activities which appeared at 72 kDa and 75 kDa (Table 5.2) as previously observed [75]. LPS injection did not significantly alter 72 kDa or 75 kDa MMP-2 plasma activities, whether in the absence or presence of doxycycline. In control animals, MMP-9 plasma activities were detected at

92 kDa and 135 kDa (the former is a full length MMP, whereas the latter is a lipocalin associated form of proMMP-9 [18]). 6 hr after LPS injection, 92 kDa and 135 kDa activities rose significantly above control levels. Doxycycline treatment significantly reduced the LPS induced increase in 92 kDa but not 135 kDa gelatinolytic activities.

5.4 Discussion

We have previously demonstrated an upregulation of MMPs in vascular hyporeactivity to LPS as well as the protective effects of MMP inhibition in *in vitro* models of endotoxemia [74]; however, the effects of *in vivo* administration of the MMP inhibitor, doxycycline, on vascular hyporeactivity caused by *in vivo* LPS are currently unknown. This study demonstrates that treatment with doxycycline *in vivo* protects against the LPS-induced development of vascular hyporeactivity to vasoconstrictors *ex vivo*. In the aortae of endotoxemic rats (with and without doxycycline) we observed increased specific MMP-2 activity versus control animals (shown as an increase in MMP-2 activity per unit protein) similar to that seen in aorta from normal rats exposed to LPS *in vivo* [74]. This upregulation may contribute to contractile dysfunction during endotoxemia. In the plasma, we observed a significant upregulation of 92 kDa MMP-9 activity which was attenuated with doxycycline. This upregulation of both MMP-2 and -9 activities in the aorta and plasma, respectively, present novel mechanisms that may contribute to vascular hyporeactivity during endotoxemia. The MMP inhibitor doxycycline was able to attenuate LPS-induced hyporeactivity to both PE and KCl, indicating a fundamental dysregulation of the contractile machinery which may in part be due to MMP activity.

Despite the large body of evidence implicating excess generation of NO as a central mediator in the development of vascular hypocontractility and hypotension in sepsis, few studies have examined the relationship between NO and MMPs. In one study of septic shock examining a cecal ligation and puncture model, MMP inhibition with a chemically modified tetracycline was able to reduce the cecal ligation and puncture-induced increase in plasma nitrate levels for 24 hr [277]. In contrast, the NO scavenger diethyldithiocarbamate *in vivo* has been shown to decrease MMP-2 and -9 protein in the plasma [278]. Here, we have shown an upregulation of iNOS in aorta from endotoxic rats; however, doxycycline administration did not alter iNOS expression. This indicates that the protective effect of MMP inhibition in our model of endotoxemia is either downstream or independent of iNOS. Possible downstream fates of NO include its spontaneous formation with $O_2^{\cdot-}$ to form $ONOO^{\cdot}$. $ONOO^{\cdot}$ has been shown to activate MMPs via oxidation of the catalytic Zn^{2+} in the active site of the enzyme [237]. NO can also bind to and activate soluble guanylate cyclase; NO and cyclic GMP have been shown to increase the expression of MMP-9 in vascular smooth muscle [279]. However, the contribution of the NO/cGMP pathway to MMP-induced vascular hyporeactivity stimulated by LPS will require further studies.

In the plasma, MMP-9 activity was induced which was attenuated by pre-treatment with doxycycline. The tertiary granules of polymorphonuclear leukocytes contain MMP-9 and are released upon LPS administration [280,281]; however the role of MMP-9 in septic shock is poorly understood. This upregulation of circulating MMP-9 may also contribute to vascular contractile dysfunction, as knockouts of this enzyme show resistance to endotoxic shock as evidenced by survival studies [196]. In various

clinical studies, increased plasma MMP-9 is found in septic patients, and the level of MMPs was correlated with the severity of sepsis [193]. Moreover, our laboratory has demonstrated a potential role for MMP-9 in cardiac contractile dysfunction in endotoxemia [73].

Symptoms of vascular hyporeactivity *in vivo* may involve an interaction with both plasma MMP-9 and vascular smooth muscle or endothelial MMP-2. We have recently investigated the role of the endothelium in the upregulation of MMP-2 activity in *in vitro* models of endotoxin shock [282]. Several studies have demonstrated the direct vasodilatory effects of MMP-2 and -9 *in vitro* which may serve as another pathophysiological mechanism in endotoxic shock [24, 25].

Despite the large body of evidence demonstrating extracellular effects of MMPs, work from our laboratory demonstrate acute intracellular effects of MMPs. Specifically, it is shown that MMP-2 can degrade troponin I in cardiac myocytes, an intracellular regulator of cardiac contraction [213]. Therefore it is tempting to assume that MMPs may act intracellularly to degrade susceptible proteins in vascular smooth muscle in septic shock. We are currently investigating potential targets of MMPs in the vasculature during endotoxemia.

These results reveal for the first time, the effects of the most potent MMP inhibitor, doxycycline, on *in vivo* LPS-induced vascular hyporeactivity to vasoconstrictors *ex vivo*. This information is important to understand the development of LPS-induced vascular hyporeactivity to vasoconstrictors *in vivo*, as the body of evidence supporting this area of research is centered on NO. Here we introduce new therapeutic targets for the treatment of vascular hyporeactivity in models of endotoxic shock.

Table 5.1 pEC_{50} values of aortic contractile responses to PE

Group	Control	Control + Doxy	LPS	LPS + Doxy
pEC_{50}	7.44 ± 0.06	7.21 ± 0.07	6.73 ± 0.17*	6.97 ± 0.10

*$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 7.

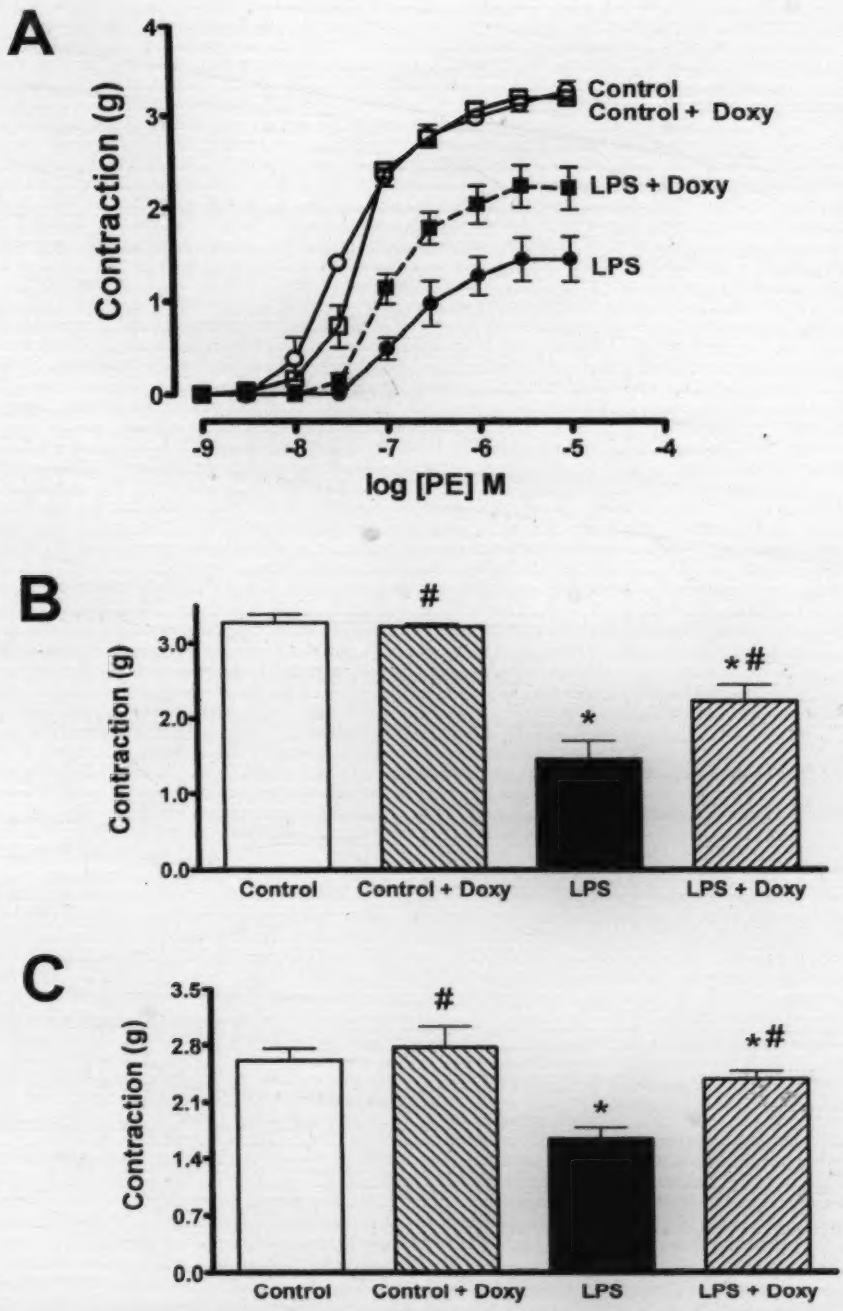


Figure 5.1 (A) Concentration-response relationship to PE (1 nM – 10 μM) in aortae isolated from the various treatment groups. (B) Quantification of maximum response to PE (10 μM). (C) Quantification of the response to KCl (75 mM). $^{*}p < 0.05$ vs Control, $^{\#}p < 0.05$ vs LPS, one way ANOVA, Neuman-Keuls post hoc test. Bars represent SEM, n = 3-7 rings/group. Experiments performed by J. Cena and M.M. Lalu.

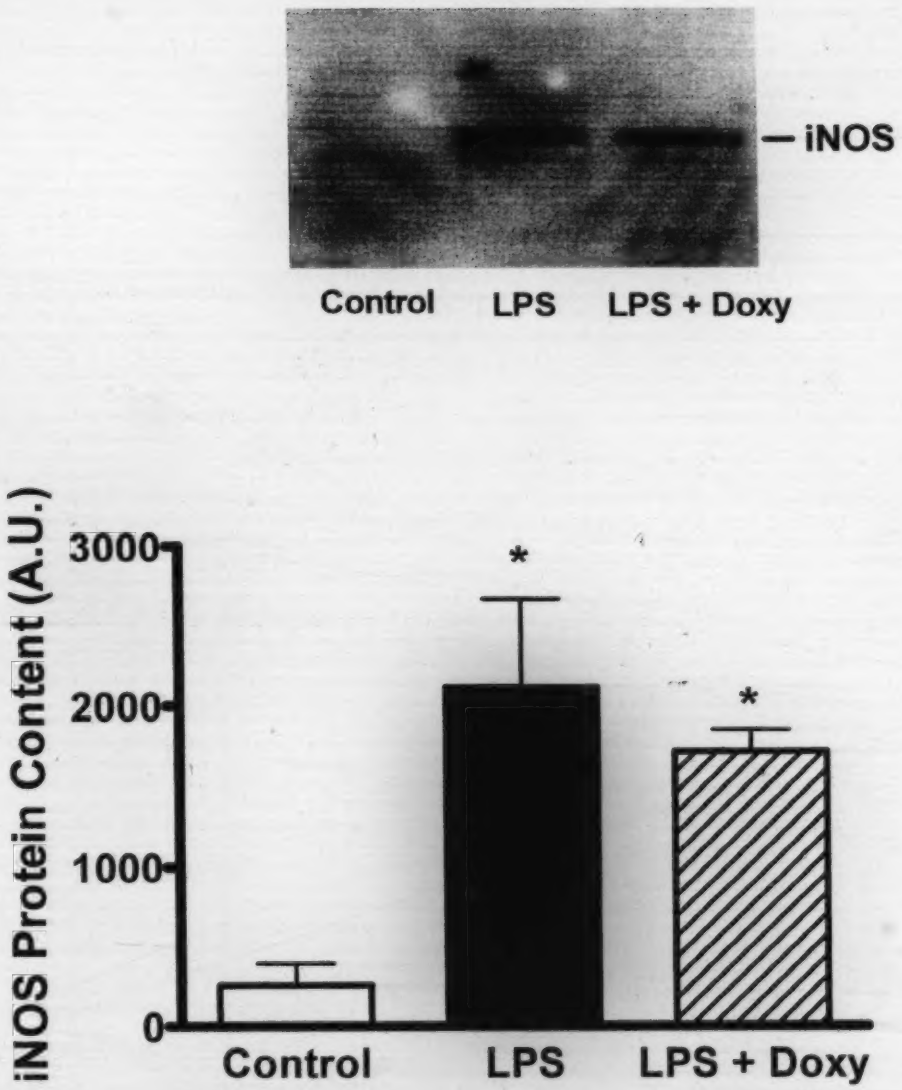


Figure 5.2 Above: Representative immunoblot for iNOS in aortic homogenate. Below: quantification of iNOS level in aortic homogenates. *$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4 homogenates/group. Experiments performed by J. Cena, M.M. Lalu, and A.K. Chow.

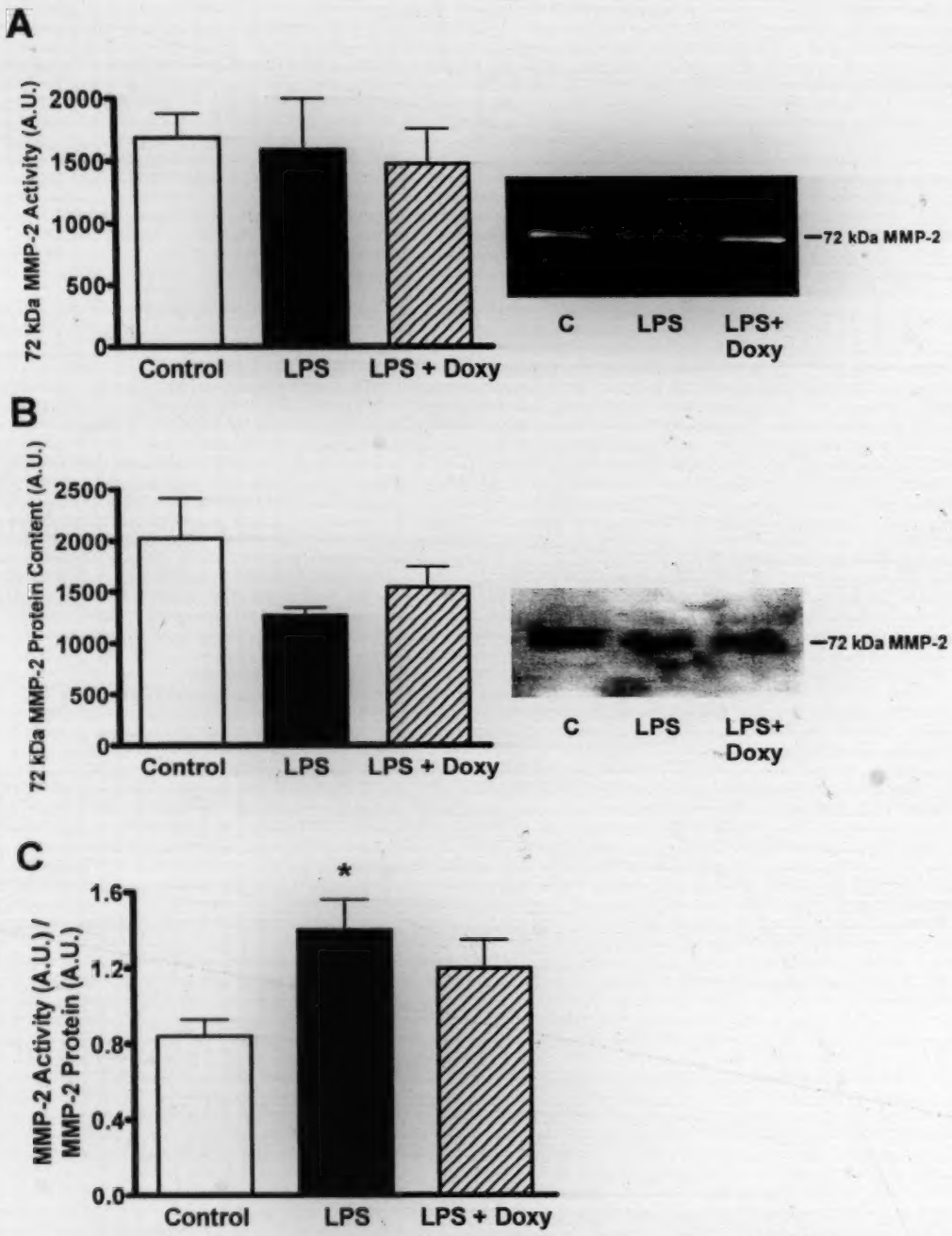


Figure 5.3 (A) Left: quantification of 72 kDa MMP-2 activity in aortae; Right: representative zymogram. (B) Left: quantification of 72 kDa MMP-2 protein; Right: representative western blot. (C) Ratio of 72 kDa MMP-2 activity / 72 kDa MMP-2 protein level. * $p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test. Bars are ± SEM, n = 4 homogenates/group. Experiments performed by J. Cena, M.M. Lalu, and A.K. Chow.

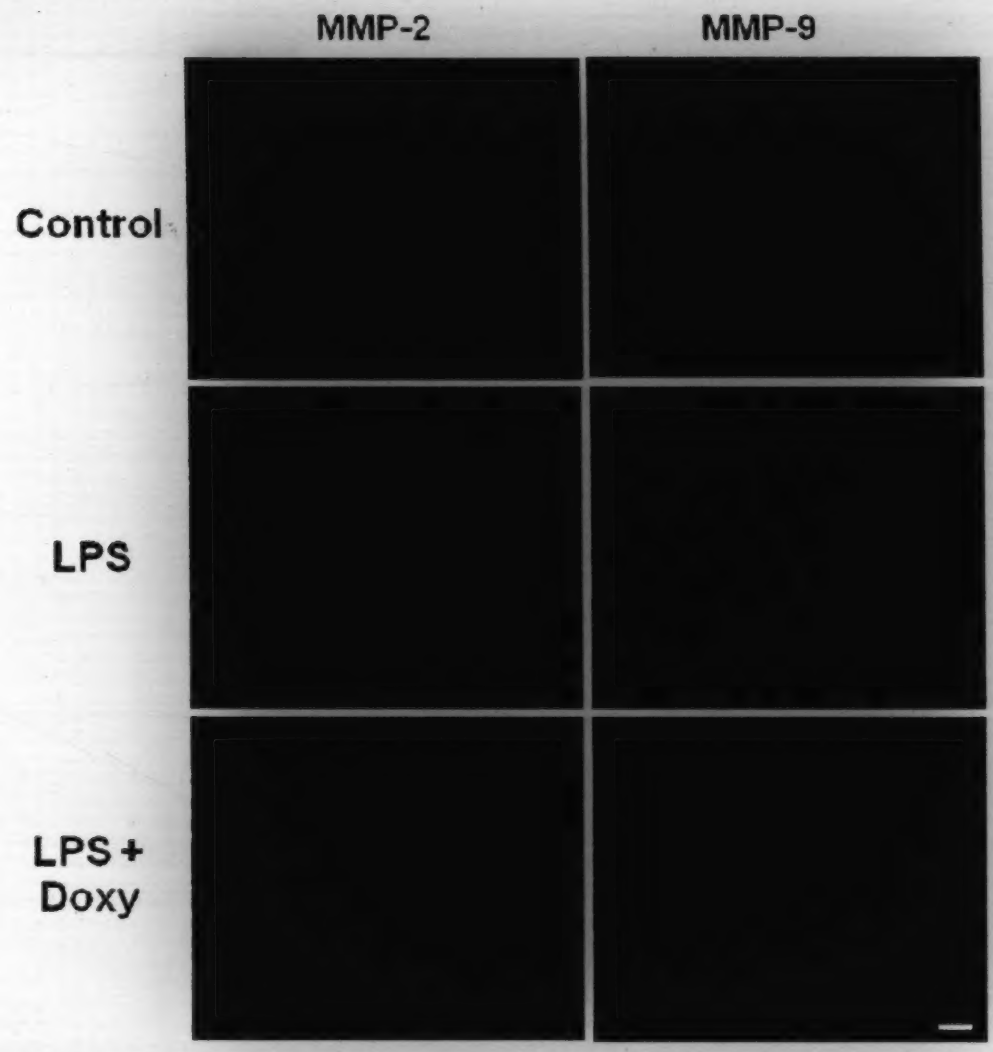


Figure 5.4 Representative immunohistochemical distribution of MMP-2 (left column) and MMP-9 (right column) in aortic cross sections from Control (top), LPS (middle), and LPS + doxycycline (Doxy, bottom) treated rats. Panels are representative of aortae from 4 rats/treatment group and processed for confocal immunohistochemical analysis which showed similar results. Red represents MMP-2 or -9 staining, blue represents nuclear (DAPI) staining. Scale bar is 20 μm for all images. Experiments performed by J. Cena, W.J. Cho, and M.M. Lalu.

Table 5.2 MMP activities in plasma using gelatin zymography

		Control	LPS	LPS + Doxy
MMP-2	**75 kDa**	109±13	120±21	92±22
	72 kDa	1205±277	1576±264	1132±277
MMP-9	**135 kDa**	99±39	1495±278*	1201±287*
	92 kDa	195±82	1750±560*	786±306$^{\#}$

*$p < 0.05$ vs Control, $^{\#}p < 0.05$ vs LPS, one way ANOVA, Neuman-Keuls post hoc test, n = 4 samples/group. Experiments performed by J. Cena, M.M. Lalu, and M. Bagdan.

CHAPTER 6
THE NO/CGMP PATHWAY CONTRIBUTES TO MATRIX METALLOPROTEINASE-INDUCED VASCULAR HYPOREACTIVITY STIMULATED BY INTERLEUKIN-1β

A portion of this chapter is in preparation. Cena, J., Rahman, S., Reimer, C., Schulz, R. The NO/cGMP pathway contributes to matrix metalloproteinase-induced vascular hyporeactivity stimulated by interleukin-1β.

6.1 Introduction

Sepsis is a debilitating clinical syndrome in which the cardiovascular symptoms dominate its clinical presentation. These are characterized as myocardial dysfunction as well as severe arterial hypotension and vascular hyporeactivity to vasoconstrictor agonists [35]. The hyporeactivity poses a severe problem for treating sepsis pharmacologically in that high doses of vasopressor agents are required to maintain an even less than adequate blood pressure. This phenomenon is due to the insensitivity of the vasculature to these agents [38]. Although several suggestions have been proposed to be involved in the development of vascular hyporeactivity, the mechanisms remain unclear.

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is involved in the pathogenesis of sepsis [283]. Clinical and basic scientific research has demonstrated that pharmacological inhibition of this cytokine reduces symptoms of septic shock [28-30]. Moreover, infusion of this cytokine induces a shock-like state in various animal models [26,284]. Additionally, incubation of blood vessels *ex vivo* with this cytokine is a well established model of vascular hyporeactivity to vasoconstrictor agonists [77,285]. Experimentally, IL-1β is capable of stimulating the expression of inducible nitric oxide synthase (iNOS) which is also believed to be central to the cardiovascular complications of septic shock [28,110,286,287].

MMPs are a family of zinc-dependent endopeptidases involved in a variety of physiological and pathophysiological processes. Classically, MMPs are synthesized as inactive zymogens. Upon cleavage of the pro-peptide domain, the catalytic site is exposed, rendering the MMP active. The gelatinases (MMP-2 and -9), a class within the MMP family, are involved in the pathophysiology of various cardiovascular diseases

[162,163,251]. We have demonstrated that inhibition of MMPs protects against LPS and IL-1β-induced vascular hyporeactivity to vasoconstrictors *in vitro* [74]. However, the pathway for MMP activation with respect to vascular hyporeactivity induced by IL-1β is not well understood. Despite the prevalence of the involvement of NO and iNOS in the development of septic shock and vascular hyporeactivity [46,58,70,71,137,270], we sought to determine the relationship of the NO/cGMP pathway and MMPs in vascular hyporeactivity using an *in vitro* IL-1β model.

6.2 Materials and Methods

Animals

Animals are described in Section 2.1.

Interleukin-1β-induced vascular dysfunction *in vitro*

To preliminarily assess the degree of IL-1β-induced depression in vascular contractility, isolated rat aortic rings were incubated for 6 hr at 37 °C with IL-1β (1, 10, and 100 ng/mL) followed by the protocol described in Section 2.4. A concentration of 10 ng/mL was used for the remainder of the study using the protocol described in Section 2.4 with the following additions: a) 30 μM GM6001 (vehicle: DMSO) or ONO-4817 (N-[(1S,3S)-1-[(Ethoxymethoxy)methyl]-4-(hydroxyamino)-3-methyl-4-oxobutyl]-4-phenoxybenzamide, ONO Pharmaceuticals, vehicle: DMSO), MMP inhibitors, to test for MMPs, b) 3 μM ODQ (1H-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one, Sigma, vehicle: 10 ddH_2O: 1 DMSO v:v) to test for soluble guanylate cyclase, c) 30 μM MnTE-2-PyP (manganese(III) tetrakis (*N*-ethylpyridinium-2-yl) porphyrin, Calbiochem, vehicle: ddH_2O), a superoxide dismutase mimetic, to test for superoxide, d) 50 μM FeTTPS

(5,10,15, 20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III], Calbiochem, vehicle: ddH_2O), a $ONOO^-$ decomposition catalyst, to test for $ONOO^-$ e) 30 μM L-NAME, a NOS inhibitor, to test for NOS. To control for the effects of DMSO, the IL-1β treated group contained 0.05% DMSO v/v. Aortic rings were placed into the culture medium after drugs were added.

In order to assess the effects of MMP, NOS, or soluble guanylate cyclase inhibition on basal MMP-2 protein expression and activity, control rings were treated with either GM6001 (30 μM), ONO-4817 (100 μM), L-NAME (30 μM), or ODQ (3 μM) and analyzed by western immunoblot and gelatin zymography as described below.

Preparation of aorta homogenate and culture media

Each aortae was split into 6 pieces and treated with one of the conditions described above. One piece was used for functional analysis and the remaining 5 pieces were frozen in liquid N_2 and homogenized as described in Section 2.7. The culture media from these 5 pieces was placed at 4 ˚C and processed and analyzed immediately to avoid freeze-thaw cycles. These samples were analyzed by western blot and gelatine zymography as described below.

Western immunoblotting analysis

The protocol is described in Section 2.10 using the following primary antibodies: an anti-mouse MMP-2 antibody (1:1000 dilution, MAB3308, Chemicon), an anti-mouse iNOS antibody (1:1000 dilution, N32020/L20, Transduction Laboratories), and an anti-rabbit actin antibody (1:500 dilution, Santa Cruz).

Measurement of MMP activity by zymography

Aortic homogenates and culture media were analyzed by gelatin zymography. This protocol is described in Section 2.9.

6.3 Results

Concentration dependence of IL-1β-induced vascular hyporeactivity

To evaluate the degree to which IL-1β induces vascular dysfunction, concentration-response curves to phenylephrine were performed on aortic rings which had been incubated for 6 hr at 37 ˚C with different concentrations of IL-1β (1, 10, and 100 ng/mL) before mounting and equilibrating them in the organ baths. Increasing concentrations of IL-1β yielded a marked concentration-dependent decrease in vascular contractility. Control rings contracted to E_{max} 3.33 ± 0.12 g, n = 3, whereas IL-1β (1 ng/mL), IL-1β (10 ng/mL), and IL-1β (100 ng/mL) contracted to 2.41 ± 0.28, 1.90 ± 0.34, and 1.65 ± 0.30 g (n = 4 for each), respectively. After incubation with IL-1β, contraction to KCl was also impaired and significantly different in IL-1β-treated groups versus control (data not shown). A concentration of 10 ng/mL IL-1β was chosen for experiments included in this study.

MMP inhibition prevents IL-1β-mediated vascular hyporeactivity

After 6 hr incubation with IL-1β, aortic rings exhibited a marked reduction in maximum contractility to PE (Control: E_{max} 2.99 ± 0.25 g, n = 9; IL-1β: E_{max} 0.75 ±0.11 g, n = 7; Figure 6.1). IL-1β also caused a rightward shift in the concentration-response curve (Control: pEC_{50} 7.12 ± 0.11, n = 9; IL-1β: pEC_{50} 6.34 ± 0.16, n = 7; Table 6.1).

Incubation of IL-1β in the presence of MMP inhibitors, GM6001 or ONO-4817, significantly reduced the IL-1β-induced depression of the maximum contraction to PE (IL-1β + ONO-4817: E_{max} 1.85 ± 0.19 g, n = 4; IL-1β + GM6001: E_{max} 1.90 ± 0.20 g, n = 4; Figure 6.1). Incubation with MMP inhibitors did not prevent the rightward shift in the concentration-response curve (IL-1β + ONO-4817: pEC_{50} 6.20 ± 0.09, n = 4; IL-1β + GM6001: pEC_{50} 6.36 ± 0.14, n = 4; Table 6.1). GM6001 or ONO-4817 alone did not affect the pEC_{50} or E_{max} of control aorta (n = 3 for each, data not shown). IL-1β treatment significantly decreased KCl-stimulated contraction which was prevented by pre-treatment with GM6001 or ONO-4817 (Table 6.4).

MMP inhibition does not affect IL-1β-mediated upregulation of aortic iNOS

To evaluate if MMP inhibition affects the expression of iNOS, we examined iNOS protein levels after homogenization of IL-1β-stimulated aortae in the presence or absence of the MMP inhibitors GM6001 or ONO-4817. IL-1β significantly upregulated iNOS protein levels; this effect was unaltered in groups treated with MMP inhibitors. iNOS was barely detectable in control vessels (Figure 6.2).

Effect of IL-1β-stimulation ± MMP inhibitors on MMP-2 protein level and activity

After 6 hr incubation with IL-1β, a trend towards a decrease in 72 kDa MMP-2 protein level was observed (Figure 6.3A). In the presence of MMP inhibitors GM6001 or ONO-4817, MMP-2 protein levels were significantly less than control. 72 kDa MMP-2 activity in aortic homogenate as analyzed by gelatin zymography was unchanged in all experimental groups (Figure 6.3B). No evidence of MMP-9 activity (92 kDa) was seen in any of the groups. Incubation of rat aorta with IL-1β resulted in a significant increase of 72 kDa MMP-2 activity in the culture medium (Figure 6.4) suggesting a release of this

enzyme from the aortae. In the presence of GM6001 or ONO-4817, 72 kDa activity in the culture medium was reduced to levels not different from controls. GM6001 or ONO-4817 alone did not affect the level of MMP-2 protein or activity in control aorta (Figure 6.5A and B), nor did these drugs affect the release of MMP-2 into the culture media (Figure 6.5C). In all groups, 64 kDa and 75 kDa MMP-2 activities were below the detection limit and non-quantifiable.

Inhibition of NOS or soluble guanylate cyclase prevent IL-1β-induced vascular hyporeactivity

The NOS inhibitor L-NAME abolished the IL-1β-mediated reduction in contractile response to PE (IL-1β + L-NAME: E_{max} 2.87 ± 0.50 g, n = 4, Figure 6.6). Furthermore, L-NAME abolished the IL-1β-mediated rightward shift in the concentration-response curve (IL-1β + L-NAME: pEC_{50} 7.12 ± 0.18, n = 4, Table 6.2). The soluble guanylate cyclase inhibitor ODQ significantly reduced the development of hyporeactivity to PE (IL-1β + ODQ: E_{max} 2.39 ± 0.19 g, n = 4). Treatment with ODQ did not prevent the IL-1β-mediated rightward shift in the concentration-response to PE (IL-1β + ODQ: pEC_{50} 6.67 ± 0.11, n = 4). L-NAME or ODQ alone did not affect the pEC_{50} or E_{max} of control aortae (data not shown). L-NAME or ODQ did not, however, prevent the IL-1β-mediated depression in KCl-contraction (Table 6.3).

Effects of FeTTPS and MnTE-2-PyP on IL-1β-induced vascular hyporeactivity

Addition of the $ONOO^-$ decomposition catalyst FeTTPS (50 μM) or the superoxide dismutase mimetic MnTE-2-PyP (30 μM) at concentrations found to be protective in cytokine-induced contractile dysfunction in the heart [132,288] did not

prevent IL-1β-mediated changes in E_{max} or pEC_{50} versus controls (Figure 6.7 and Table 6.4, respectively).

Effect of IL-1β-stimulation ± L-NAME or ODQ on MMP-2 protein level and activity

IL-1β did not alter the level of 72 kDa MMP-2 in aortic homogenates; however, inhibition of NOS or sGC significantly reduced basal MMP-2 levels in both control and IL-1β-treated vessels (Figure 6.8A). Consistent with this reduced level of MMP-2 protein, 72 kDa MMP-2 activity was significantly decreased in homogenates of aortae treated with L-NAME or ODQ (Figure 6.8B). L-NAME or ODQ alone did not affect the expression or activity of MMP-2 in control aortae (Figure 6.5A and B), nor did these drugs affect MMP-2 release into the culture media (Figure 6.5C). 64 kDa and 75 kDa MMP-2 activities were below the detection limit and non-quantifiable in all groups.

Inhibition of NOS and sGC prevent MMP-2 activation

After stimulation with IL-1β, a significant increase in MMP-2 activity was observed in the culture medium. Treatment of aortae with L-NAME or ODQ prevented this release (Figure 6.9), indicating a role for NO and cGMP in IL-1β-stimulated release of MMP-2 activity. L-NAME or ODQ alone did not affect the release of MMP-2 in control aortae (Figure 6.5C). 64 kDa and 75 kDa MMP-2 activities were below the detection limit and non-quantifiable.

Effects of KCl-mediated contractions on experimental groups

75 mM KCl was added to assess for intact electromechanical coupling mechanism in the aorta (Table 6.3). Control aortae contracted to 3.10 ± 0.14 g (n = 6), whereas after IL-1β incubation, the contractions were significantly impaired (IL-1β: 1.89 ± 0.14 g, n = 9). Incubation with MMP inhibitors significantly prevented the IL-1β-stimulated

depression in KCl response (IL-1β + GM6001: 2.74 ± 0.10 g, n = 14; IL-1β + ONO-4817: 3.00 ± 0.10 g, n = 3). Interestingly, L-NAME and ODQ did not prevent against IL-1β-mediated contractile depression (IL-1β + L-NAME: 1.91 ± 0.20 g, n = 4; IL-1β + ODQ: 2.09 ± 0.10 g, n = 4).

6.4 Discussion

Our laboratory has previously shown that MMP inhibitors protect against vascular hyporeactivity induced by IL-1β; however, the mechanism is currently unknown. Here we present a novel mechanism demonstrating a biochemical pathway for MMP-2 activation which contributes to vascular hyporeactivity stimulated by IL-1β. Consistent with our previous investigation [74], MMP inhibitors prevented IL-1β-induced vascular hyporeactivity. Interestingly, MMP inhibition did not alter IL-1β-mediated upregulation of iNOS protein level. Furthermore, IL-1β treatment resulted in an increased MMP-2 activity in the surrounding culture media. Since a large body of evidence supports the involvement of NO and sGC in the development of IL-1β-mediated vascular hyporeactivity to vasoconstrictors [57,289], we therefore tested the effects of L-NAME and ODQ on MMP protein levels and activity. A decrease in MMP-2 protein level and activity was observed in the aortae of IL-1β-stimulated aortae incubated with L-NAME or ODQ. This was accompanied by a decrease in MMP-2 activity in the culture medium versus control. Interestingly, control aortae treated with only L-NAME or ODQ showed no reduction in aortic MMP-2 protein levels or gelatinolytic activity. This indicates a possible mechanism of MMP-2 downregulation that is dependent upon the presence of

IL-1β. Moreover, control aortae treated with only L-NAME or ODQ showed no difference in the release of MMP-2 in the surrounding incubation medium.

Incubation with MMP inhibitors significantly reduced the IL-1β-induced decrease in maximum contractile response to phenylephrine. IL-1β also caused a marked right shift in the concentration-response curve, indicating a decrease in the potency of α-adrenoceptor stimulation; however, incubation with MMP inhibitors did not prevent this rightward shift. These results indicate that the protective effects of MMP inhibition on IL-1β-induced vascular hyporeactivity are mediated by changes in the efficacy of α-adrenergic vasoconstriction and not changes in potency.

Aortic MMP-2 levels did not change in IL-1β-treated aortae; however, in the presence of MMP inhibitors, MMP-2 levels decreased significantly. Possible reasons for this include the ability of chemically modified tetracyclines (which possess MMP inhibitory activity in the absence of antibacterial effects) to decrease MMP-2 gene expression, and therefore, protein expression [290,291]. However, aortic homogenate MMP-2 activity was unchanged in all experimental groups. Interestingly, control aortae treated with GM6001 or ONO-4817 alone showed a slight trend towards decreased MMP-2 protein level and activity, however, this was non-significant. Due caution should be exercised in interpreting zymographic data from tissues treated with MMP inhibitors as these drugs can dissociate from the MMP enzyme during gel electrophoresis. Consistent with our previous results [74], the release of MMP-2 into the surrounding culture media was significantly upregulated upon stimulation with IL-1β, while incubation with MMP inhibitors brought this effect down to levels not significantly different from control. This suggests a possible extracellular effect of MMP-2 on the

development of vascular hyporeactivity to vasoconstrictors as well as a mechanism for MMP-2 release that may be prevented by pharmacological inhibition of MMPs.

It is interesting to note that aortae treated with IL-1β in the presence of MMP inhibitors show no alterations in iNOS protein expression, indicating that the protective effects of MMP inhibition on IL-1β-mediated vascular hyporeactivity are downstream or independent of this enzyme. One potential fate of NO produced from iNOS could include its spontaneous formation with superoxide to form $ONOO^-$. Although evidence in the literature supports the involvement of $ONOO^-$ in the development of vascular hyporeactivity to vasoconstrictors [72,270,292,293], we have incubated a $ONOO^-$ decomposition catalyst or a superoxide dismutase mimetic at concentrations previously shown to be protective in a cytokine model of cardiac dysfunction in rat hearts [133,288] and found no improvement in contractile depression in IL-1β-stimulated aortae. Another potential fate of NO is its classical activation of sGC which results in vascular relaxation. We therefore tested the effect of L-NAME and ODQ on MMP protein levels and activity.

As expected, incubation of IL-1β-stimulated aortae with L-NAME and ODQ significantly protected against the depression in maximum contractility to vasoconstrictors, indicating that NO and sGC are important factors in this process. L-NAME prevented the rightward shift in the concentration-response curve, indicating that the shift in potency of α-adrenergic stimulation is mediated by NO. Interestingly, incubation with ODQ did not alter the potency of α-adrenergic vasoconstriction in IL-1β-treated vessels. Thus suggesting different mechanisms of action on vascular hyporeactivity by NO and cGMP.

Upon examination of MMP-2 protein levels of IL-1β-treated vessels in the presence of L-NAME and ODQ, it is revealed that inhibition of NOS and sGC decrease MMP-2 protein to levels significantly below the control and IL-1β treated vessels. Interestingly L-NAME and ODQ did not affect basal MMP-2 protein levels in control aortae. This decrease in MMP-2 protein levels reflects the significant decrease in MMP-2 activity seen in these aortae. This is also consistent with the MMP-2 activity in the surrounding culture media, as L-NAME and ODQ treatment reduced the IL-1β-mediated release of MMP-2. A downregulation of MMP-2 protein level in the aortae may decrease the pool of MMP-2 available to be released.

It is interesting to note that a decrease in MMP-2 protein levels in the presence of the MMP inhibitors was only observed in the presence of IL-1β while the same vessels exhibited no changes in MMP-2 activity. Along the same lines, a decrease in MMP-2 protein and activity after incubation with L-NAME and ODQ was observed only in the presence of IL-1β, as control aortae treated with these compounds showed no differences in basal MMP-2 protein levels and activity.

In contrast to our results, one group has examined cultured rat vascular smooth muscle cells as well as *ex vivo* aortic explants and found that inhibition of NOS upregulates MMP-9 expression within the aortic wall [294,295]. However, in a rat model of endotoxemia, NO was shown to contribute to LPS-induced gastric injury via a MMP-2-dependent mechanism [296]. Interestingly the NO donor, 2-(N,N-diethylamino)-diazenolate-2-oxide, and 8-Br-cGMP were found to upregulate MMP-13 and -9 mRNA and protein levels in cultured bovine endothelial [297,298] and rat aortic vascular smooth muscle cells [279], respectively. One group demonstrated a biphasic effect of NO on

MMP-9 inhibition in murine macrophages; at lower concentrations, NO was found to enhance MMP-9 activity via a cGMP-dependent mechanism, whereas higher concentrations of NO resulted in an inactivation of MMP-9 activity [299]. Despite the current literature, MMP-9 was undetectable in the aorta or the culture medium in our zymographic analysis.

MMP inhibition significantly prevented the depression in KCl mediated contractions indicating a disruption in electromechanical coupling. However, incubation with L-NAME or ODQ did not prevent this decline. This is an interesting observation suggesting that electromechanical and pharmacomechanical mechanisms with respect to MMPs and NO/cGMP inhibition are pharmacologically distinct.

MMPs and their potential roles in septic shock, including vascular hyporeactivity to vasoconstrictors, represent a new frontier in the pharmacology of septic shock. This study explores further mechanisms of MMP-mediated vascular hyporeactivity stimulated by IL-1β. Excessive production of NO as well as an activation of sGC are established pathophysiological mechanisms of septic shock and vascular dysfunction [57,289]; however, this study suggests that MMPs may be also involved in this pathway. Our laboratory is currently investigating potential targets of these enzymes.

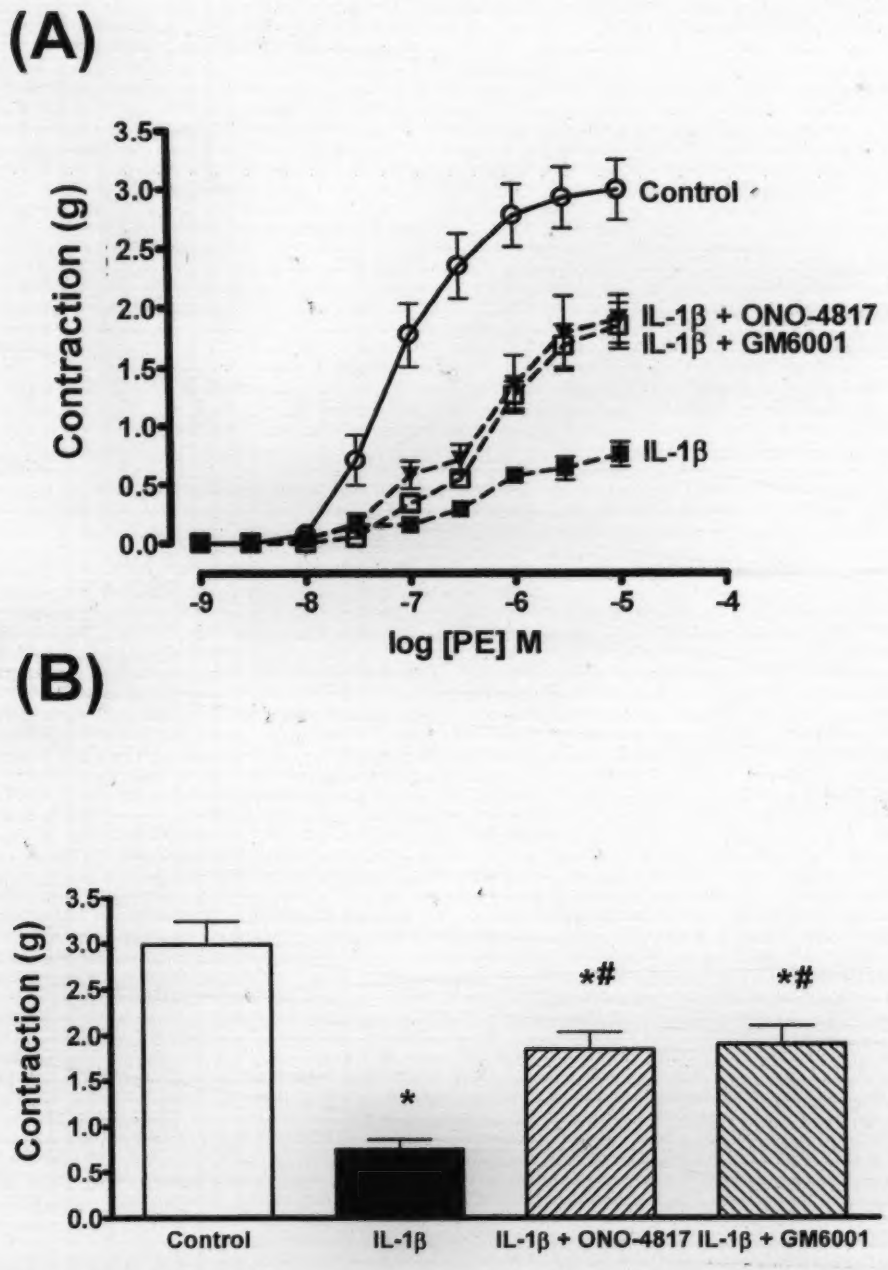
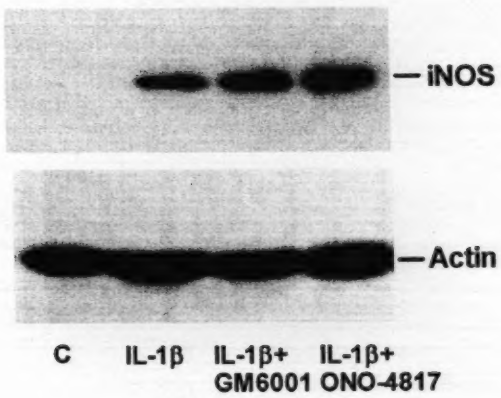


Figure 6.1 (A) Contractile response of aortic rings from normal rats to increasing concentrations of PE following 6 hr incubation at 37 °C in the presence or absence of IL-1β (10 ng/mL) ± GM6001 (30 μM) or ONO-4817 (100 μM). (B) Quantification of maximum PE contraction (*$p < 0.05$ vs Control; #$p < 0.05$ vs IL-1β, one way ANOVA, Neuman-Keuls post hoc test, n = 4 aortic rings/group).

Table 6.1 pEC_{50} values of aortic contractile responses to PE

Group	Control	IL-1β	IL-1β + ONO-4817	IL-1β + GM6001
pEC_{50}	7.12 ± 0.11	6.33 ± 0.16*	6.20 ± 0.09*	6.36 ± 0.14*

*$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4-9.



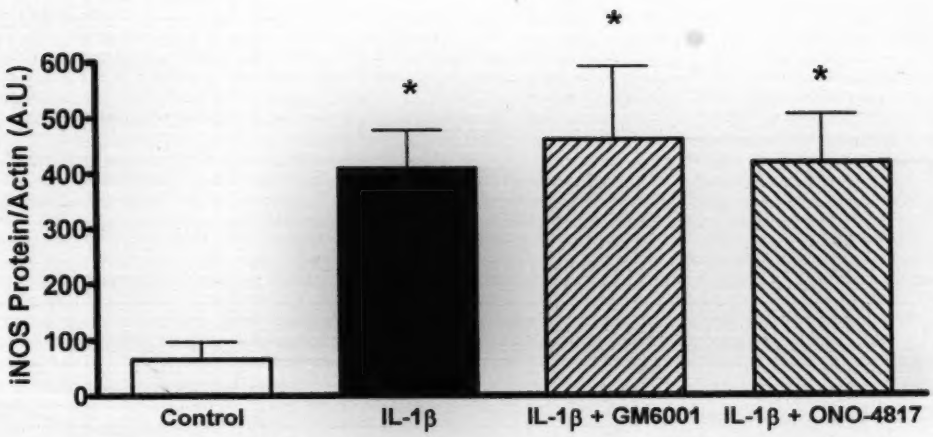


Figure 6.2 Above: A representative western blot of vascular homogenate iNOS protein levels following treatment with IL-1β (10 ng/mL) ± GM6001 (30 μM) or ONO-4817 (100 μM). Below: Quantification of iNOS protein levels (*$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4 aortic rings/group).

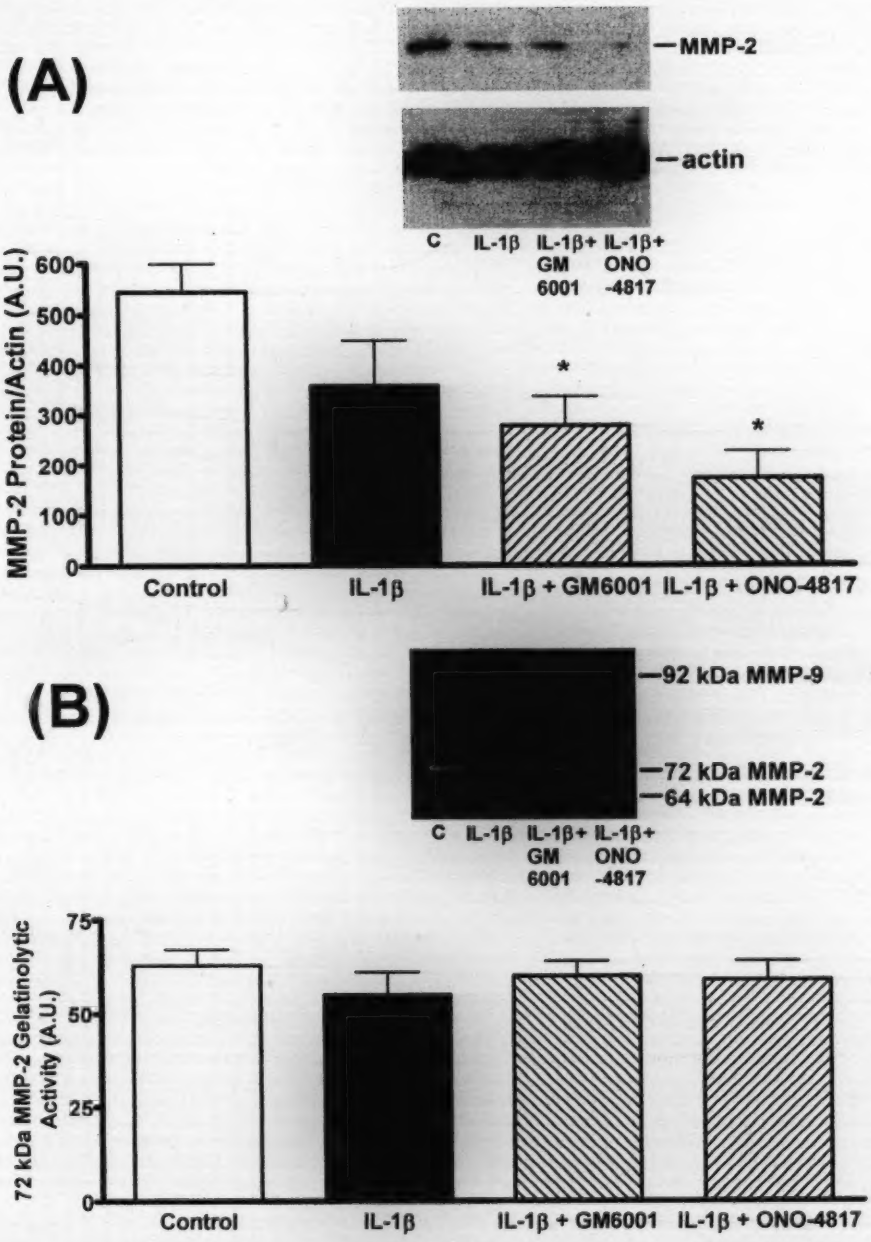


Figure 6.3 (A) Above: a representative western blot of vascular homogenate MMP-2 levels following treatment with IL-1β (10 ng/mL) ± GM6001 (30 μM) or ONO-4817 (100 μM). Below: quantification of MMP-2 protein levels. (B) Above: a representative zymogram of vascular homogenate gelatinolytic activities. Below: quantification of 72 kDa MMP-2 gelatinolytic activity. *$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4 rings/group.

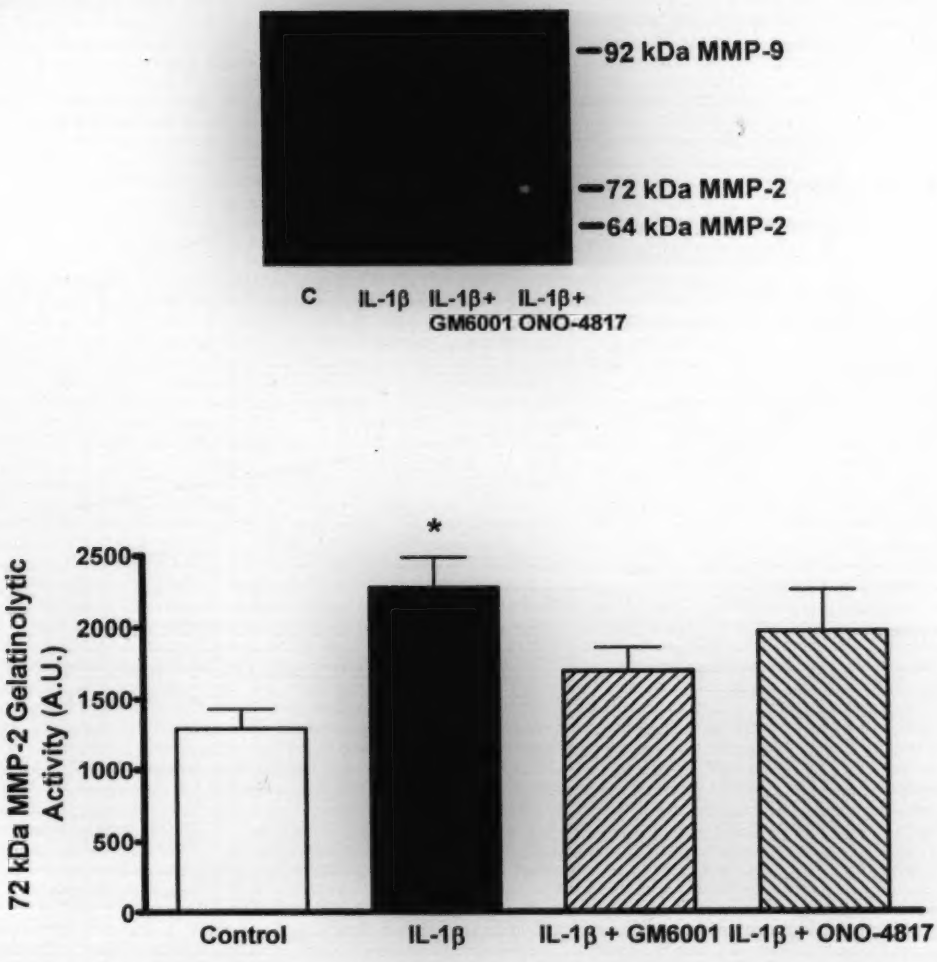


Figure 6.4 Above: representative zymogram of culture media from aortas incubated with IL-1β (10 ng/mL) ± GM6001 (30 μM) or ONO-4817 (100 μM). Below: quantification of 72 kDa MMP-2 gelatinolytic activity (*$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4 culture media samples/group).

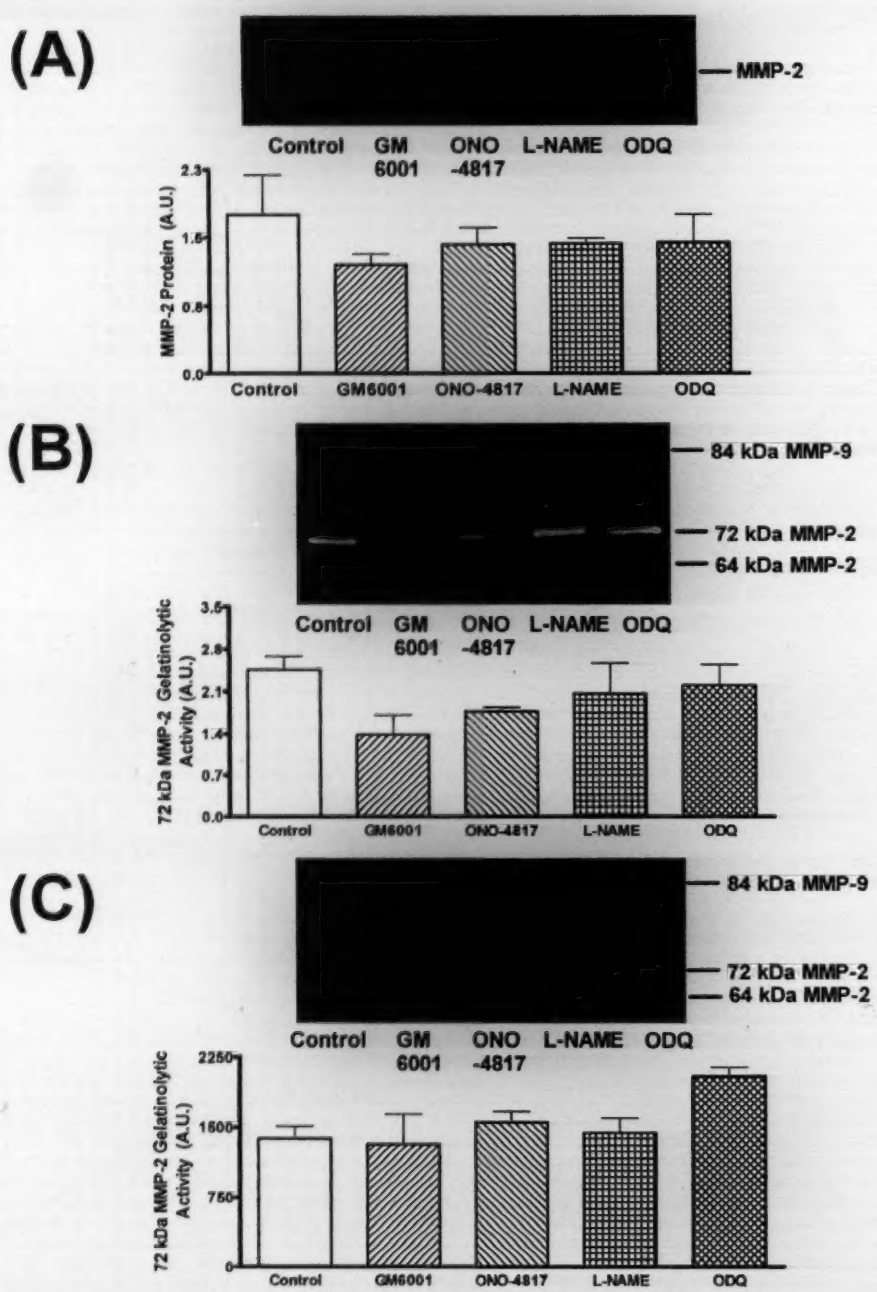


Figure 6.5 Control aortae treated with GM6001 (30 μM) or ONO-4817 (100 μM) or L-NAME (30 μM) or ODQ (3 μM) at 37 ˚C for 6 hr. (A) Above: a representative western blot of vascular homogenate MMP-2 levels. Below: quantification of MMP-2 protein levels. (B) Above: a representative zymogram of vascular homogenate gelatinolytic activities. Below: quantification of 72 kDa MMP-2 activity. (C) Above: a representative zymogram of gelatinolytic activity from surrounding culture medium. Below: quantification of 72 kDa MMP-2 activity. n = 3 aortae/group.

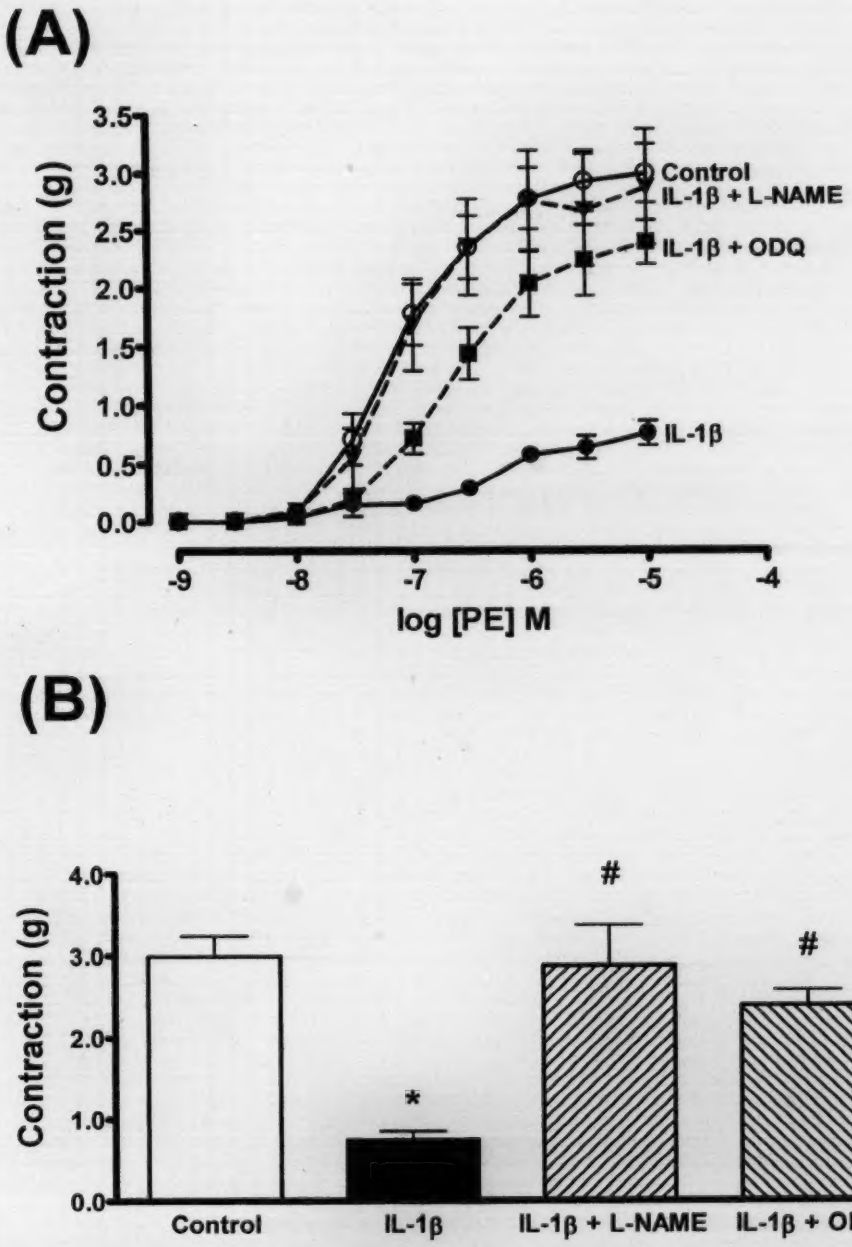


Figure 6.6 (A) Contractile response of aortic rings from normal rats to increasing concentrations of PE following 6 hr incubation at 37 ˚C in the presence or absence of IL-1β (10 ng/mL) ± L-NAME (30 μM) or ODQ (3 μM). (B) Quantification of maximum PE contraction (*$p < 0.05$ vs Control, #$p < 0.05$ vs IL-1β, one way ANOVA, Neuman-Keuls post hoc test, n = 4 aortic rings/group).

Table 6.2 pEC_{50} values of aortic contractile responses to PE

Group	Control	IL-1β	IL-1β + L-NAME	IL-1β + ODQ
pEC_{50}	7.12±0.11	6.33±0.16*	7.12±0.09$^{\#}$	6.67±0.11

*$p < 0.05$ vs Control, $^{\#}p < 0.05$ vs IL-1β, one-way ANOVA, Neuman-Keuls post hoc test, n = 4-9 rings/group.

Table 6.3 Contraction of IL-1β-treated aortae in response to 75 mM KCl

Group	Contraction (g)
Control	3.10 ± 0.11
IL-1β	1.89 ± 0.14*
IL-1β + GM6001	2.74 ± 0.10#
IL-1β + ONO-4817	3.04 ± 0.10#
IL-1β + L-NAME	1.91 ± 0.20*
IL-1β + ODQ	2.09 ± 0.10*

*$p < 0.05$ vs Control, #$p < 0.05$ vs IL-1β, one-way ANOVA, Neuman-Keuls post hoc test, n = 3-9 rings/group.

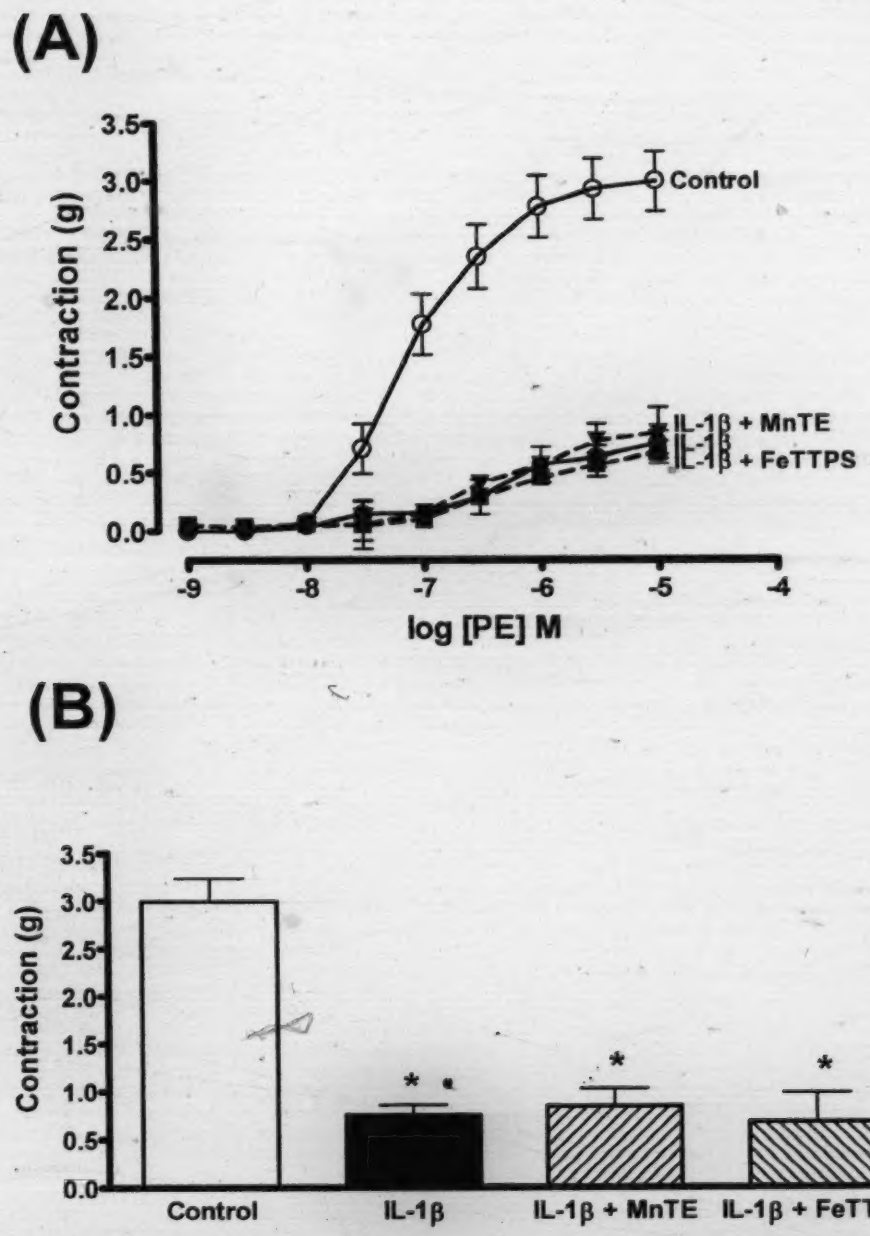


Figure 6.7 (A) Contractile response of aortic rings from normal rats to increasing concentrations of PE following 6 hr incubation at 37 °C in the presence or absence of IL-1β (10 ng/mL) ± MnTE-2-PyP (MnTE, 30 μM) or FeTTPS (50 μM). (B) Quantification of maximum PE contraction (*$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 3-9 aortic rings/group).

Table 6.4 pEC_{50} values of aortic contractile responses to PE

Group	Control	IL-1β	IL-1β + MnTE	IL-1β + FeTTPS
pEC_{50}	7.12 ± 0.11	6.33 ± 0.16*	6.44 ± 0.27*	6.23 ± 0.30*

*$p < 0.05$ vs Control, one-way ANOVA, Neuman-Keuls post hoc test, n = 3-9 rings/group.

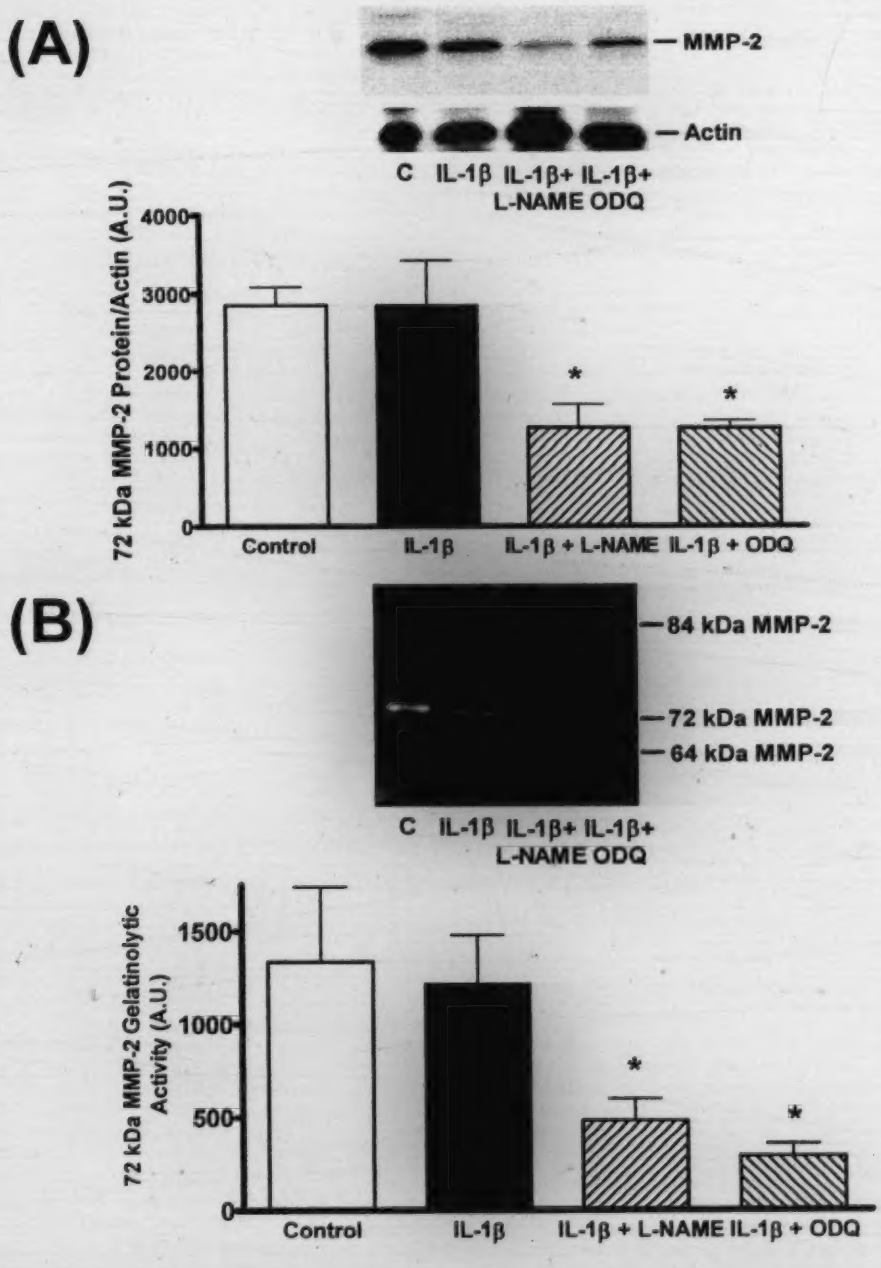


Figure 6.8 (A) Above: a representative western blot of vascular homogenate MMP-2 levels following treatment with IL-1β (10 ng/mL) ± L-NAME (30 μM) or ODQ (3 μM). Below: quantification of MMP-2 protein levels. (B) Above: a representative zymogram of vascular homogenate gelatinolytic activities. Below: quantification of 72 kDa MMP-2 gelatinolytic activity. * $p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4 rings/group.

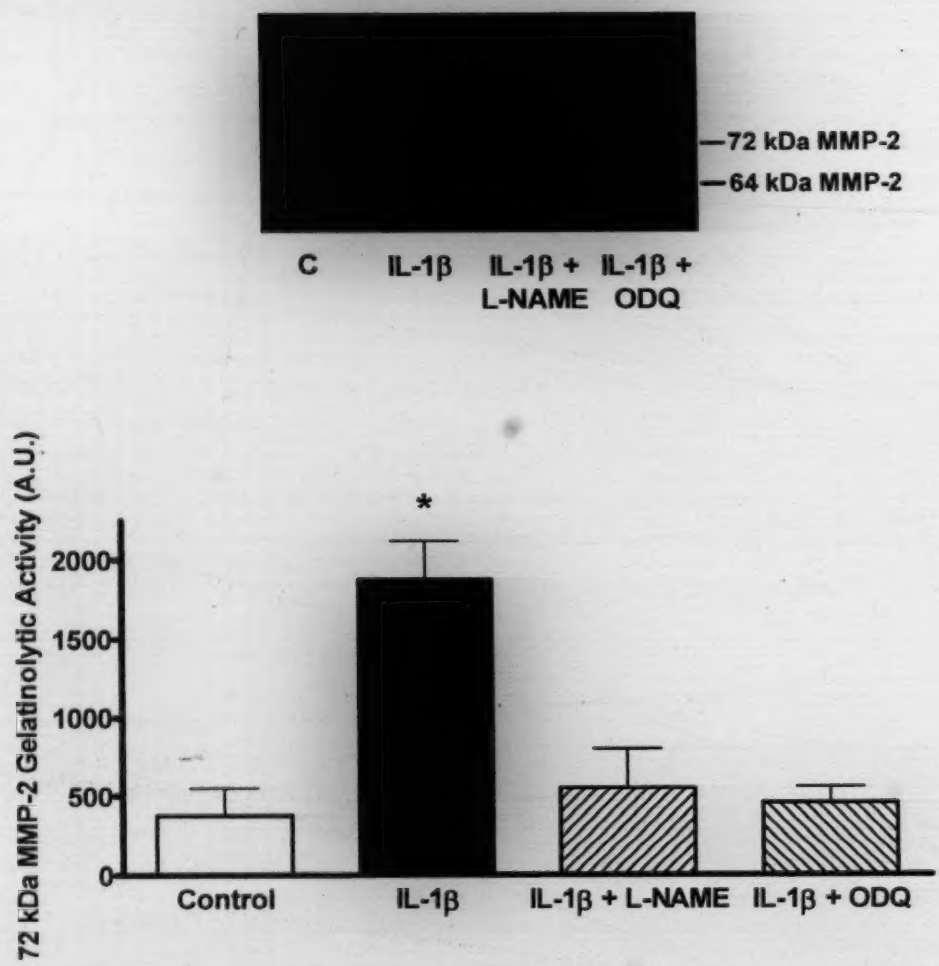


Figure 6.9 Above: representative zymogram of culture media from aortas incubated with IL-1β (10 ng/mL) ± L-NAME (30 μM) or ODQ (3 μM). Below: quantification of 72 kDa MMP-2 gelatinolytic activity (*$p < 0.05$ vs Control, one way ANOVA, Neuman-Keuls post hoc test, n = 4 culture media samples/group).

CHAPTER 7
DISCUSSION

7.1 Conclusions

As the mortality rate of clinical septic shock fails to decline, a greater understanding of the pathogenesis of this disease is essential before new drug therapies can be implemented. Since the cardiovascular symptoms of septic shock dominate its clinical presentation, understanding this facet of the disease is essential. One of the clinical manifestations of septic shock is the development of persistent arterial hypotension and involves the interaction of an excessive production of vasodilatory substances and vascular hyporeactivity to vasoconstrictor agents. Although much is known about the production of vasodilatory substances, little is known about the intrinsic inability of the vasculature to respond to vasoconstrictors in septic shock. Matrix metalloproteinases (MMPs) have received tremendous attention in recent years as these enzymes have been discovered as major players in many cardiovascular diseases. As such, I have examined the roles of MMPs in the development of vascular hyporeactivity in endotoxic shock.

In Chapter 3, I first examined MMPs in three models of shock. In an *in vitro* experimental model, inhibition of MMPs activity was found to protect against lipopolysaccharide (LPS)-mediated vascular contractile dysfunction in isolated rat aorta, thus indicating a role for MMPs in this phenomenon. I further utilized an *in vivo* LPS model, termed endotoxemia, on the changes in MMP activities in the rat aorta. We discovered increased MMP-2 and -9 activities relative to protein levels which coincided with a reduction in TIMP-4 levels. Moreover, we discovered that the MMP inhibitor, doxycycline, given *in vitro* acutely reversed the hypocontractility to PE of aorta taken from endotoxemic rats. Lastly, since cytokines also play a large role in the pathogenesis

of endotoxic shock, and MMPs have been shown to be involved in the proteolytic processing of cytokine precursors, I examined the role of MMPs downstream of this process using aortae stimulated with interleukin-1β (IL-1β) *in vitro*. Here I discovered an increase of MMP-2 activity released into the culture media of IL-1β-stimulated aortae as well as the protective effects of MMP inhibition in protecting against vascular hyporeactivity in this model. This initial study set the stage for the following chapters of this thesis by introducing the novel relationship between MMPs and the development of vascular hyporeactivity in these models of endotoxic shock.

Because of the integral role of the endothelium in controlling vascular tone, as well as orchestrating some aspects of the immune system, discovering the role of MMPs with respect to the endothelium was crucial to understanding the development of vascular hyporeactivity to vasoconstrictors. In Chapter 4 I utilized isolated rat aortae stimulated by LPS *in vitro* and examined the protective effects of the MMP inhibitor, doxycycline, in the presence and absence of the endothelium. The endothelium is well known to exacerbate LPS-induced vascular hyporeactivity to vasoconstrictors [257]; however, the contribution of MMPs in this phenomenon was unknown. My results were consistent with the existing literature, demonstrating that the endothelium contributes to LPS-mediated vascular hyporeactivity [257]. Additionally, the protective effects of the MMP inhibitor, doxycycline, were only seen in the presence of the endothelium, indicating that it may act as an origin of MMP-mediated vascular contractile dysfunction. Moreover, these results were consistent with gelatin zymograms demonstrating an upregulation of LPS-stimulated MMP-2 activity in endothelium-intact aortae, but not endothelium-denuded aortae.

In Chapter 5, in order to assess a more biologically relevant model of endotoxic shock, LPS was administered to rats *in vivo* 6 hr prior to *ex vivo* functional analysis of the aorta. Because of the pathophysiological response to LPS, administration of an MMP inhibitor *in vivo* would serve as a more clinically relevant model. We found that *in vivo* administration of the MMP inhibitor doxycycline, given 0.5 hr after LPS, prevented the development of vascular hyporeactivity to vasoconstrictors measured *ex vivo*. Biochemical analysis of these aortae revealed enhanced MMP-2 activity relative to its protein level, further supporting the notion that MMPs are involved in vascular hyporeactivity. Despite the large body of evidence supporting inducible nitric oxide synthase (iNOS) as a principal mediator of vascular hyporeactivity in endotoxemia [123], doxycycline did not affect the LPS-stimulated increase in iNOS protein levels suggesting that MMPs may act downstream or independent of this key enzyme.

Although the immune system protects against foreign invaders, a malfunctioning or overactive immune system is deleterious to the host. In septic shock, the body produces a cascade of inflammatory mediators which are key drivers of vascular contractile dysfunction. Failure of anti-cytokine trials may stem from a lack of understanding of the mechanism of action of these pro-inflammatory mediators. Cytokines are capable of stimulating a complex cascade of other immunogens thus further complicating the pathogenesis of septic shock [25]. In Chapter 6, in order to understand the relationship between IL-1β and MMPs in the development of vascular hyporeactivity to vasoconstrictors, we stimulated aortae with IL-1β *in vitro* in order to pharmacologically dissect this relationship. We utilized two other MMP inhibitors, GM6001 and ONO-4817, in order to strengthen the evidence that MMPs are involved in

vascular hyporeactivity to vasoconstrictors stimulated by IL-1β. In agreement with our previous data, MMP inhibitors protected against IL-1β-mediated vascular hyporeactivity to vasoconstrictors. Moreover, inhibition of MMPs activity did not affect the expression of iNOS, indicating that the protective effects of MMP inhibition *in* vitro are downstream or independent of this key enzyme. Consistent with data obtained in Chapter 3 I discovered increased MMP-2 activity in the culture media of IL-1β-stimulated vessels. Moreover, this effect was blocked upon MMP inhibition. Inhibition of either MMPs, NOS, or soluble guanylate cyclase prevented the IL-1β-induced vascular hyporeactivity. However, only inhibition of MMPs protected from the IL-1β-induced hyporeactivity to KCl, suggesting distinct mechanisms of action on electromechanical versus pharmacomechanical contractile mechanisms. Furthermore, inhibition of NOS or soluble guanylate cyclase completely abolished IL-1β-mediated activation of MMP-2. This indicates that MMPs may exert their effects downstream of the NO/cyclic GMP pathway and contribute to vascular hyporeactivity to vasoconstrictors. These observations reveal the therapeutic potential of MMP inhibitors. Since NOS inhibitors have not shown benefit in clinical trials, inhibition of MMPs may be a novel therapeutic strategy. Although we have discovered that the NO/cyclic GMP pathway and MMPs play a role in the development of vascular hyporeactivity, the downstream effectors must be examined. Cyclic GMP activates its associated protein kinase (protein kinase G) which results in phosphorylation of proteins involved in vascular smooth muscle relaxation. The involvement of this kinase with respect to MMPs is an important piece of the puzzle in elucidating the pathway for MMP-induced vascular hyporeactivity.

7.2 Limitations

One of the major limitations of these studies is the inability to unequivocally discern which member(s) of the MMP family contributes to the vascular contractile dysfunction. The MMP inhibitors utilized in these studies are broad-spectrum inhibitors that have different specificities and inhibitory constants against the MMPs. Therefore, one cannot exclude the possibility of these agents interacting with MMPs other than MMP-2. I chose to focus on the gelatinases (MMP-2 and -9) because these enzymes are a major focus in various cardiovascular pathologies including septic shock [162,300] and genetic knockouts of MMP-9 are resistant to endotoxic shock [197].

Another limitation involves the use of zymography as an assay for gelatinolytic activity. Due to the reducing conditions of the gel electrophoresis protocols, inactive and active gelatinases are considered to be dissociate from any inhibitory complexes with TIMPs, thus these results should theoretically represent total potential gelatinolytic profile of a given sample. However, upon zymographic analysis of aortic homogenate I observed greater total gelatinolytic activity aftertreating the samples with aminophenylmercuric acetate, a well known chemical activator of MMPs, versus untreated controls (data not shown). Specifically, aminophenylmercuric acetate activates MMPs without a concomitant change in molecular weight [301]. Therefore we believe gelatin zymography to be a net activity assay for MMP-2 and -9.

The various models utilized throughout these investigations have each their own limitations. By virtue of their design, *in vitro* experiments are fundamentally limited in their interpretations; observations *in vitro* may not reflect results seen *in vivo*. *In vitro* experiments are designed such to limit the contribution of confounding variables

including physiological compensatory mechanisms and other non-specific processes of pharmacological agents within an intact organism. Our experiments utilizing LPS and IL-1β *in vitro* only serve as models for what might occur in an intact physiological system. Despite these limitations, results from these *in vitro* experiments provide insight into the mechanisms of LPS or IL-1β-induced vascular hyporeactivity.

LPS is present on the outer coat of gram negative bacterium and is responsible for sparking the immunological cascade seen in gram-negative sepsis. Administration of this macromolecule produces pathophysiologically distinct effects in contrast to clinical septic shock; as such, many scientists have dismissed scientific models that utilize it because of discordances between endotoxemia and clinical septic shock [302]. One group believes that endotoxemia fails to reproduce the capillary leak in septic shock [303]; however, this same group agrees that endotoxemia parallels some aspects of the disease, including the cardiovascular symptoms. A large number of treatments have proven beneficial in endotoxemia but have failed in clinical trials of septic shock [6,34]. Despite these limitations, utilizing LPS in various experimental models has revealed an extensive amount of knowledge regarding the pathogenesis of the disease because of existing parallels in symptoms of animal models and humans. Furthermore, LPS administration mimics many facets of the inflammatory response as well as the cardiovascular manifestations (including vascular hyporeactivity to vasoconstrictors) of the disease. One can conclude that endotoxemia is suitable for investigating many, but not all features of septic shock. The cecal ligation and puncture model is a more widely accepted model of septic shock [32], which many believe to be more applicable to septic shock in humans.

However, this procedure is technically demanding and is susceptible to a large user-dependent and animal-dependent variability.

It is well established that septic shock is characterized by a pathophysiological reduction in blood pressure. However, the vessel that we have chosen to study, the rat thoracic aorta, is not a major determinant of physiological blood pressure. The contractile state of the resistance and venous vasculature is a much greater determinant of blood pressure than that of the aorta. I am aware of this limitation in my investigations, and, as such, my studies have focused on the intrinsic hyporeactivity of vascular smooth muscle to vasoconstrictor agents and not the state of pathophysiological septic shock itself. My decision to adopt the aorta in models of vascular hyporeactivity is supported by evidence in the literature which demonstrate parallels of this model with vascular hyporeactivity in clinical septic shock [57-61,304]. The aorta has been employed in various key scientific discoveries of the 20th century including the discovery of endothelium-derived relaxing factor/NO [86]. Since these studies are the first to investigate the novel relationship between MMPs and LPS or IL-1β-induced vascular hyporeactivity to vasoconstrictors we believed that characterizing this biochemical relationship was best achieved using a consistent and reproducible model that offers ample protein for simultaneous biochemical and functional analysis.

7.3 Future directions

The most important avenue to pursue after completion of this thesis is the discovery of a proteolytic target of MMPs within the vasculature that contributes to vascular hyporeactivity in endotoxemia. Although I have started preliminary work in

elucidating potential targets, the question of whether or not calponin is a molecular target *in vivo* remains open. Moreover, determining which MMP(s) are involved in the proteolysis of potential targets remains to be discovered. The lack of specific MMP inhibitors has hindered scientific progress in MMP biology.

Because the aorta is a reactive conduit vessel and does not contribute to overall blood pressure, these experiments should be repeated using other vascular beds such as the mesenteric vasculature. Experiments using smaller arteries that have a greater effect on the setting of blood pressure may bring to light the therapeutic potential of MMP inhibitors for use in clinical septic shock trials.

As stated before, a more widely accepted model of septic shock is the cecal ligation and puncture (CLP) model; however, due to its variability, it was excluded from my investigations. If a reproducible model of CLP could be established, experiments presented in this thesis could also be applied. Some groups have investigated MMP inhibition in CLP [277,305] and discovered a reduction in mortality. However, these groups have examined other aspects of the disease and not the functional state of the vasculature following MMP inhibition after CLP.

The notion of pathological activation of K^+ channels during septic shock has received considerable attention in recent years concerning vascular hyporeactivity to vasoconstrictors [58,304]. The underlying scientific basis for these investigations involves the abnormal activation of K^+ channels which contribute to vascular smooth muscle cell hyperpolarization, thus leading to relaxation. Recently a pilot study was performed using a K_{ATP}-selective blocker of K^+ channels in patients with septic shock [61]. Although the trial did not show an improvement in vascular hyporeactivity

symptoms in septic patients, a possible failure of this trial may be due to the decision to use a selective K_{ATP} channel blocker. It has been suggested that instead of K_{ATP}-channels acting as the driver for vascular smooth muscle cell hyperpolarization, small conductance and big conductance Ca^{2+}-activated K^+ channels may play a greater role [60]. A future study pertaining to our work may involve the characterization of the relationship between MMPs and the activity of various K^+ channel subtypes. In a study of varicose vein formation in rats, one group has already demonstrated that large conductance Ca^{2+}-activated K^+ channels are activated upon stimulation with exogenous MMP-2 [181].

An important activation mechanism for MMPs has recently been implicated in various cardiovascular diseases [162,163]. This novel mechanism involves the activation of MMPs via peroxynitrite ($ONOO^-$). It is tempting to speculate that this mechanism may occur because increased nitrosative stress and activation of MMPs occurs in various models of septic shock. Additionally, this activation may occur intracellularly or extracellularly, thus complicating the search for potential targets of MMPs. Elucidating if whether this process occurs and the spatial location of MMP activation will aid in the determination of MMP targets in the future.

Other mechanisms to regulate MMPs activity could be possible. Our laboratory recently discovered that the phosphorylation state of MMP-2 affects its activity [306]. Specifically, phosphorylation of MMP-2 was shown to decrease its proteolytic activity. The state of MMP-2 phosphorylation in vascular smooth muscle and its ability to regulate vascular tone require further investigation. Moreover, we have recently discovered an inhibitory association of caveolin-1 with MMP-2 [162]. Interestingly, caveolin-1 has been shown to colocalize with MMP-2 in endothelial cells [260]. Therefore it would be

logical to assume a loss of caveolin-1 (which was shown to occur in LPS-stimulated macrophages [261]) may result in increased MMP-2 activity and contribute to the contractile dysfunction in septic shock.

In conclusion, results from this thesis highlighted the involvement of MMPs in the pathogenesis of septic shock. Studies succeeding these will reveal more about the role and regulation of these proteases in septic shock. To date, there have been no clinical trials examining MMP inhibition in septic shock. Investigating the development of the cardiovascular dysfunction in septic shock is crucial to understanding the disease clinically, as cardiovascular symptoms dominate its presentation. This thesis brings to light the therapeutic potential of MMP inhibition as well as the mechanisms of action of MMPs which contribute to this critical illness.

APPENDIX: ADDITIONAL PRELIMINARY STUDIES

Although I have introduced in this thesis a novel role of matrix metalloproteinases (MMPs) in contributing to vascular hyporeactivity to vasoconstrictors in the rat aorta caused by LPS *in vitro* or *in vivo* or by IL-1β *in vitro*, not much is known about the regulation, activation, and most importantly, potential targets of MMPs in the vascular wall that contribute to this process. In this appendix, I have briefly explored various avenues including the potential role of membrane microparticles in the development of MMP-induced vascular hyporeactivity in endotoxemia and potential proteolytic targets of MMPs within the vasculature. This appendix also contains experiments investigating the effects of genetic ablation of MMP-2 on vascular hyporeactivity to vasoconstrictors.

A1.1 Introduction: membrane microparticles

Membrane microparticles (MP) are shed from the plasma membrane of various cell origins including platelets, leukocytes, and endothelial cells [307]. These small vesicles (1-2 μm in diameter) are known to contain MMPs including MMP-2, -9, and MT1-MMP [203]. Their release from human monocytes is upregulated following stimulation with various cytokines or LPS [204]. Recent evidence implicates membrane derived MPs in the development of various cardiovascular inflammatory conditions including preeclampsia and septic shock [205-209]. Interestingly, MPs of endothelial origin have been implicated in the development of vascular hyporeactivity in preeclampsia [206,210-212]. Therefore, it is tempting to assume a role of MPs in the development of vascular hyporeactivity in septic shock. This study is an important follow up to Chapter 4, in which I discovered that the endothelium contributes to MMP-induced vascular hyporeactivity in response to LPS. From these results one can hypothesize that

endothelial MPs containing MMPs may be released from the endothelium following LPS stimulation, and this pathological event contributes to vascular hyporeactivity to vasoconstrictors.

A1.2 Methods

Animals

Animals are described in Section 2.1.

Rat endotoxemia

The protocol for rat endotoxemia is described in Section 2.5.

Extraction of plasma

Plasma was obtained immediately after sacrifice via intracardiac puncture with a 17 gauge needle in 0.3% sodium-citrate and centrifuged at 1494 g for 10 min at 4 ˚C. In order to assess if vascular hyporeactivity was induced, aortae were examined functionally as described below. The remaining aortic sections were snap frozen in liquid N_2 for further biochemical analysis.

Protocol for microparticle isolation

Plasma from control and LPS-injected rats (6 hr timepoint) were prepared for isolation of MPs. Isolation of MPs were performed as described with slight modifications [308,309]. Plasma was aliquoted into 250 µL eppendorf tubes and spun at 17,570 g for 30 min at 4 ˚C. 225µL of the supernatant was removed and used as an internal control. The pellet was resuspended in 200 µL of Ca^{2+}-free Krebs buffer and respun at 17570 g for 0.5 hr at 4 ˚C. The supernatant (175 µL) was discarded and the remaining pellet, known to

contain MPs [309], was diluted to 200 μL with Dulbecco's modified Eagle's medium and stored at 4 °C.

Protocol for incubation of aorta with microparticles

Untreated male Sprague-Dawley rats (250 – 350 g) were sacrificed and aortae were rapidly excised, washed and placed in Dulbecco's modified Eagle's medium (with 1000 mg/L glucose, pyroxidine HCl, $NaHCO_3$, Sigma) supplemented with 100 U/mL streptomycin, 100 U/mL penicillin (Gibco) and 50 U/mL gentamycin (Sigma). Aortae were dissected under a tissue culture hood. Two rings were cut (5 mm in length) from each aorta and placed in fresh cell culture medium (2.0 mL) with either of the following conditions: 10% v/v of the supernatant from the centrifugation of plasma from control rats described above, 10% v/v of the supernatant from the centrifugation of plasma from LPS-injected rats described above, the MP fraction from the plasma of control rats, or the MP fraction from the plasma of LPS-injected rats. The MP and supernatant fractions taken from one rat were used in the incubations of two 5 mm rings cut from another rat. Rings were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 6 hr rings were removed from the cell culture medium and mounted in organ baths for functional analysis.

Assessment of contractile function of aortae

This protocol is described in Section 2.6 with the following additions: in order to assess if MP fractions caused vascular hyporeactivity to vasoconstrictors via a MMP-dependent mechanism, GM6001 (30 μM) was added to the culture media before aorta were incubated for 6 hr at 37 °C.

Gelatin zymography

MMPs in the MP and supernatant fractions of plasma from control and LPS-injected rats were measured by zymography as described in Section 2.9.

Flow cytometric analysis of microparticle fractions

50 μL of microparticle fractions from control or LPS-injected rats were put into Eppendorf tubes and incubated in the presence or absence of the following antibodies: an anti-human annexin V antibody conjugated to FITC (1:20 v/v, VAA-33, Bender Medsystems), a biotinylated anti-rat CD62E antibody (1:10 v/v, BAF977, R&D Systems) which required a 0.5 hr incubation with a streptavidin-phycoerythrin conjugate (1:5 v/v, F0040, R&D Systems, streptavidin-phycoerythrin binds:biotinylated anti-rat CD62E antibody (4:1 molar ratio)) in order to amplify the fluorescent signal, an anti-rat CD31 antibody conjugated to Alexafluor 647 (Alexa, 1:10 v/v, TLD-3A12, AbD Serotec). Samples were incubated for 0.5 hr in the dark at room temperature with agitation. After incubation, samples were diluted with 500 μL phosphate buffered saline, placed in Falcon tubes (12x75 mm) and analyzed using a BD FACSCalibur flow cytometer. MPs were identified by their forward and side scatter. Endothelial MPs resulting from their immunological activation were defined as CD62E positive, whereas endothelial MPs resulting from apoptosis were defined as CD31 positive [310]. Positive annexin V staining is representative of all membrane MPs [311].

A1.3 Results

Flow cytometric analysis of membrane MP fractions

Plasma of one control and one LPS-treated rat were analyzed for antigen specific staining of MP markers. MP fractions incubated without conjugated antibodies exhibited no autofluorescence and demonstrate forward and side scatter (data not shown). Comparing the MP fractions stained with anti-annexin V-FITC, there appears to be greater positive staining for annexin V in the MP fractions from the plasma of LPS-treated rats versus control (Control: Q4 Top-left; LPS: Q4 Bottom-left; Figure A1.1). Fluorescence was observed after incubation of MP fractions with anti-CD62E-Phyco; however, there appears to be no difference in the MP fractions from the plasma of control or LPS-treated rats (Control: Q1 Top-left; LPS: Q1 Bottom-left; Figure A1.2). In comparing MP fractions incubated with anti-CD31-Alexa, there appears to be increased fluorescence in MP fractions from the plasma of LPS-treated rats versus control (Control: Q4-1 Top-right; LPS: Q4-1 Bottom-right; Figure A1.3). Positive events are summarized in Table A1.1 where FITC, Phyco, and Alexa events are from their respective quadrants (Control: FITC Q4; Phyco Q2; Alexa Q4-1; LPS: FITC Q4; Phyco Q2; Alexa Q4-1).

Functional analysis of aortae incubated with MP fractions

The same MP fractions prepared as described above were also used to induce vascular hyporeactivity to vasoconstrictors in rat aortae. Aortae which had been incubated with the supernatant and MP fractions from the LPS-treated rats exhibited no significant changes in the pEC_{50} values versus controls (Control supernatant, 7.43 ± 0.08; Control MP, 7.31 ± 0.12; LPS supernatant, 6.83 ± 0.27; LPS MP, 6.64 ± 0.37; n = 4 for each). On the other hand, the E_{max} was depressed in aortae incubated with the supernatant or MP fractions of the plasma from LPS treated rats versus those incubated with supernatant or MP fractions from the plasma of control rats (Control supernatant, 2.89 ±

0.14 g; Control MP, 3.02 ± 0.31 g; LPS supernatant, 0.98 ± 0.19 g; LPS MP, 1.32 ± 0.33 g; n = 4 for each, Figure A1.4). In a preliminary study, aortae incubated with MP fractions from LPS-treated rats in the presence of the MMP inhibitor GM6001 (30 μM) showed a greater contractile response to PE versus those aortae treated with vehicle alone (Figure A1.5).

Zymographic analysis of supernatant and MP fractions

After centrifugation of plasma from control and LPS-treated rats, the supernatant and MP fractions of the plasma from these groups were subjected to gelatin zymography. Zymographic analysis revealed a significant upregulation of 135 kDa and 84 kDa MMP-9 activities in the supernatant from LPS-treated rats versus control as well as a significant upregulation of 135 kDa, 92 kDa, and 84 kDa MMP-9 activities in the MP fraction from LPS-treated rats versus control (Figure A1.6). Due to the much higher level of MMP-2 activity, densitometric analysis of MMP-2 associated gelatinolytic activities could not be completed.

A1.4 Discussion

The generation of membrane MPs in endotoxemia may represent a novel pathophysiological mechanism for the development of vascular hyporeactivity to vasoconstrictors. Although more experiments are necessary, there appears to be an upregulation of all general membrane MPs as evidenced by an increase in FITC staining in the MP fractions from the plasma of LPS-injected rats versus control. Furthermore, there appears to be an increase in Alexa staining (a marker for CD31, an antigen present

in MPs from apoptotic endothelial cells) in the MP fractions of plasma from LPS-treated rats. As CD31 is a marker for endothelial MPs, this may indicate a possible upregulation of endothelial MPs which contributes to vascular hyporeactivity. This result is consistent with the current literature demonstrating that MPs of endothelial origin contribute to vascular hyporeactivity [212,308,312]. Despite the evidence regarding MPs, there is a considerable discussion concerning the identity, purification, and generation of MPs in the current literature [313]. Further experiments are required to determine if it is indeed the MPs that contribute to vascular hyporeactivity in our model of endotoxic shock.

Functional analysis of aortae revealed a depression in E_{max} and rightward shift in the EC_{50} in the aortae incubated with the supernatant and MP fractions from LPS-treated rats. This indicates a reduction in the efficacy and potency of α-adrenergic vasoconstriction. The observed decrease in E_{max} and EC_{50} in the supernatant fractions of plasma from LPS-treated rats may be due to the absence of wash steps in centrifugation which would serve to purify the MP fraction, or the presence of cytokines or LPS contaminants in the supernatant fractions. Zymographic analysis of the supernatant and MP fractions revealed a significant upregulation in MMP-9 activity. The source of MMP-9 could possibly be from polymorphonuclear leukocytes [280,281] or MPs. Further experiments are needed to unequivocally determine if MMPs contained within MPs in the plasma contribute to vascular hyporeactivity to vasoconstrictors.

A2.1 Introduction: calponin as a potential proteolytic target of MMP-2

Potentially the most valuable information pertaining to this area of study is the discovery of MMP targets in endotoxemia and septic shock. In recent years, MMPs have

been discovered to play a role in a number of cardiovascular pathologies [162,163]. Our laboratory has discovered that MMP-2 has an intracellular role during myocardial ischemia and reperfusion injury. Specifically, MMP-2 was found to degrade an intracellular regulator of cardiac myocyte contraction, troponin I [213], and this event contributes to contractile dysfunction of the heart as a result of ischemia and reperfusion injury. Therefore, I decided to examine potential intracellular targets of MMP-2 relevant to smooth muscle cells and their susceptibility to cleavage by MMP-2.

Calponin is a 34 kDa actin-binding protein that is homologous to troponin I [214] and is located in the cytoskeleton and the contractile apparatus of smooth muscle cells. Traditionally the role of calponin was once thought to be the inhibition of myosin ATPase activity [215]; however, new roles in its regulation of vascular contractility are currently being investigated. The calponin molecule contains distinct motifs that define its structure. From the amino terminal end, 45 residues comprise one of the two binding sites for Ca^{2+}-binding proteins such as calmodulin; this is followed by a so-called "calponin" homology domain [314] which is present in a large number of actin cross-linking proteins and signalling molecules [315]. *In vitro* experiments demonstrate that this domain contains the site responsible for the inhibition of myosin ATPase [316]. The affinity of calponin for actin can be modulated by its phosphorylation at Ser 175 [317], however, whether this is significant *in vivo* remains to be determined. Calpain, an ubiquitous intracellular protease, is capable of cleaving calponin [216]. There may be some discrepancies in the literature, however, because calpastatin, a known calpain inhibitor, may also inhibit MMPs (Schulz lab – unpublished observations). This

observation may question the validity of various studies involving calpain in that some of the observed results may be attributable to MMPs.

There is a discussion in the current literature investigating the physiological effects of calponin. Interestingly, rats subjected to hypoxia exhibited an increase in calponin which was directly proportional to the degree of vascular hyporeactivity to vasoconstrictors [318]. In agreement with its ability to inhibit myosin ATPase activity, calponin has been shown to reduce unloaded isometric forces and to reduce shortening velocity [319-321]. Conversely, calponin has also been shown to increase the contractile response to various vasoconstrictors including noradrenaline and phenylephrine via a protein kinase C-dependent mechanism [322-325]. Specifically, in aortic smooth muscle, stimulation with noradrenaline and phenylephrine resulted in a redistribution of calponin from the cytosol to the plasma membrane. Thus, hypothetically, its cleavage and dysregulation would result in a decreased contractile response. Moreover, expression of calponin differs among rat strains, and those with calponin (Sprague-Dawley) have a higher sensitivity to α-adrenergic vasoconstriction versus those without calponin (Kyoto-Wistar) [322]. Calponin may regulate smooth muscle contraction independently of actinomyosin interactions. It has been shown to function as a signalling protein to facilitate extracellular signal-related kinase-dependent signalling [325]. As stated above, calponin has been shown to translocate to the membrane upon stimulation with vasoconstrictor agonists [323,326]. In support of its function as a signalling protein, calponin was found to colocalize with the mitogen activated protein kinase suggesting its involvement in bovine tracheal smooth muscle cell proliferation [324]. Another group found that calponin is involved in suppressing cell attachment and transition into various

stages of the cell cycle in cultured smooth muscle cells [327]. These studies suggest that cleavage or dysregulation of calponin may serve as a vascular smooth muscle cell signal for its differentiation from a contractile to a synthetic phenotype.

A2.2 Materials and methods

Purified recombinant human calponin (1.3 μg or 5 μg, a kind gift from Michael Walsh, University of Calgary) or human recombinant troponin I (2 μg, a kind gift from Danuta Szczesna-Cordary, University of Miami) was incubated with human recombinant active 64 kDa MMP-2 (10 ng, Oncogene; 562:1 troponin I:MMP-2 molar ratio; 919:1 calponin:MMP-2 molar ratio) for 2 hr in 50 mmol/L Tris-HCl buffer (5 mM $CaCl_2$ and 150 mM NaCl) at 37 °C. In order to determine MMP-2-mediated proteolysis, the reaction mixture was preincubated with the MMP inhibitor *o*-phenanthroline (10 μM) for 15 min at 37 °C before adding calponin. The reaction mixtures (total volume 40 μL) were analyzed by 12% SDS-PAGE under reducing conditions and proteins were visualized by coomassie blue staining.

Rat endotoxemia

The protocol for rat endotoxemia is described in Section 2.5.

Interleukin-1β-induced vascular dysfunction *in vitro*

Isolated rat aortic rings were incubated for 6 hr at 37 °C with IL-1β as described in Section 2.4.

Preparation of aorta homogenates

This protocol is described in Section 2.7.

Western immunoblotting analysis

Western immunoblotting analysis is described in section 2.10 using the following primary antibody: a home-made rabbit polyclonal calponin antibody directed against the full length chicken calponin protein (courtesy of Michael Walsh, University of Calgary).

A2.3 Results

In vitro degradation of human recombinant calponin

We tested purified human recombinant calponin for its susceptibility to proteolysis by human recombinant MMP-2 (Figure A2.2). Calponin was degraded after 2 hr incubation with MMP-2. Preincubation of MMP-2 with *o*-phenanthroline prevented the degradation of calponin. This observation was repeatable over several experiments. Troponin I was run parallel to calponin as a positive control for MMP-2 activity.

Analysis of calponin levels in aortae from LPS-treated rats

The level of calponin was determined in aortae from LPS-injected rats (the same ones utilized for the isolation of MP fractions). We observed a trend (*$p = 0.10$) towards an increase in calponin in aortae from LPS-treated rats versus control (Figure A2.3).

Analysis of calponin from aortae stimulated with IL-1β-mediated vascular hyporeactivity *in vitro*

We examined calponin protein levels from IL-1β-treated rat aortae *in vitro* in one experiment (Figure A2.4). This revealed a possible increase in calponin levels over control after IL-1β treatment which was abrogated with the MMP inhibitor GM6001.

A2.4 Discussion

We observed a degradation of calponin by MMP-2 *in vitro*, however, in aortae treated with IL-1β *in vitro* and from LPS-treated rats we observed a trend towards an increase in calponin levels. If indeed calponin was cleaved *in situ*, this increase may be a compensatory mechanism; this can be examined by measuring calponin mRNA by quantitative polymerase chain reaction analysis. Along the same lines, a compensatory mechanism has been implicated in cultured rat cardiomyocytes utilizing a model of doxorubicin-induced cardiac injury. Specifically, this group examined the susceptibility of titin, a large myofibrillar protein, to proteolysis via the enzyme calpain, and revealed that immunohistochemical analysis of rat cardiomyocytes subjected to doxorubicin revealed no changes in titin levels; however, western immunoblotting revealed an increase in protein levels [328]. Currently, a controversy exists concerning the biological role of calponin; one study demonstrates that an increase in calponin levels in the rat aorta result in a decrease in contractile response to vasoconstrictors [318], while some studies show that a lower level of calponin is associated with a decreased contractile response in aorta isolated from rats and ferrets [322,324,325]. Further studies are needed to assess if calponin is cleaved during endotoxemia and if this event contributes to vascular hyporeactivity to vasoconstrictors.

A3.1 Introduction: genetic ablation of MMP-2

Genetic knockouts of enzymes provide valuable information because such investigations eliminate confounding variables associated with the specificity of pharmacological inhibitors. However, due to compensatory mechanisms, the

interpretations of such studies require due caution. Concerning MMP KO studies, there is one study which has examined the effects of ablation of MMP-9 in a model of septic shock. It revealed that MMP-9 KO mice are resistant to endotoxic shock in terms of survival [196]. The effect of MMP-2 ablation on vascular hyporeactivity to vasoconstrictors in shock has not yet been studied. Since I have implicated potential roles for MMP-2 in the development of vascular hyporeactivity in endotoxemia, I assessed the dependence of MMP-2 on IL-1β-mediated vascular hyporeactivity in a MMP-2 KO mouse model.

A3.2 Materials and methods

Assessment of vascular reactivity in mouse thoracic aortae

This protocol is similar to Section 2.4 with the following changes: two aortic rings (2 mm in length) from MMP-2 KO and wild-type (WT) mice (C57BL/6J, 12-16 months old) were mounted and a resting tension of 0.9 g was applied during equilibration.

A3.3 Results

IL-1β-induced vascular hyporeactivity to PE in aortae from MMP-2 KO mice

Aortic rings from WT mice exhibited a greater response to PE versus KO mice (Figure 7.10). Aortic rings from WT animals had a higher maximum contraction (E_{max}) versus IL-1β-treated rings (WT Control, 0.73 ± 0.08 g, n = 3; WT IL-1β, 0.14 ± 0.03 g, n = 3). Control and IL-1β-treated rings from MMP-2 KO mice exhibited a decreased E_{max} compared to WT Control (KO Control, 0.14 ± 0.03 g, n = 4; KO IL-1β, 0.09 ± 0.02 g, n = 3). The pEC_{50} for aortas from each of the groups were not significantly different from

each other (WT Control, 6.14 ± 0.26, n = 3; WT IL-1β, 5.85 ± 0.14, n = 3; KO Control, 6.07 ± 0.31, n = 4; KO IL-1β, 6.33 ± 0.20, n = 3).

The contraction to 75 mM KCl exhibited a similar pattern to that of PE amongst experimental groups in that aortae from WT controls contracted to significantly higher E_{max} versus all other groups (n = 3-4 per group, Figure A3.1).

A3.4 Discussion

Aortae from MMP-2 KO mice exhibited vascular hyporeactivity to PE as well as a depressed contraction to KCl without prior stimulation with IL-1β, indicating a fundamental dysregulation in contractile mechanisms. From these results it would be tempting to speculate that MMP-2 is required for intact pharmaco- and electromechanical coupling mechanisms; however, absence of this enzyme may result in an abnormal phenotype as evidenced by murine embryogenesis studies which suggest its importance in normal development [329,330]. However, one study outside our laboratory revealed no histological differences between WT and MMP-2 KO aorta [331]. Taken together, this model appears to be inappropriate for *in vitro* studies of vascular hyporeactivity to vasoconstrictors.

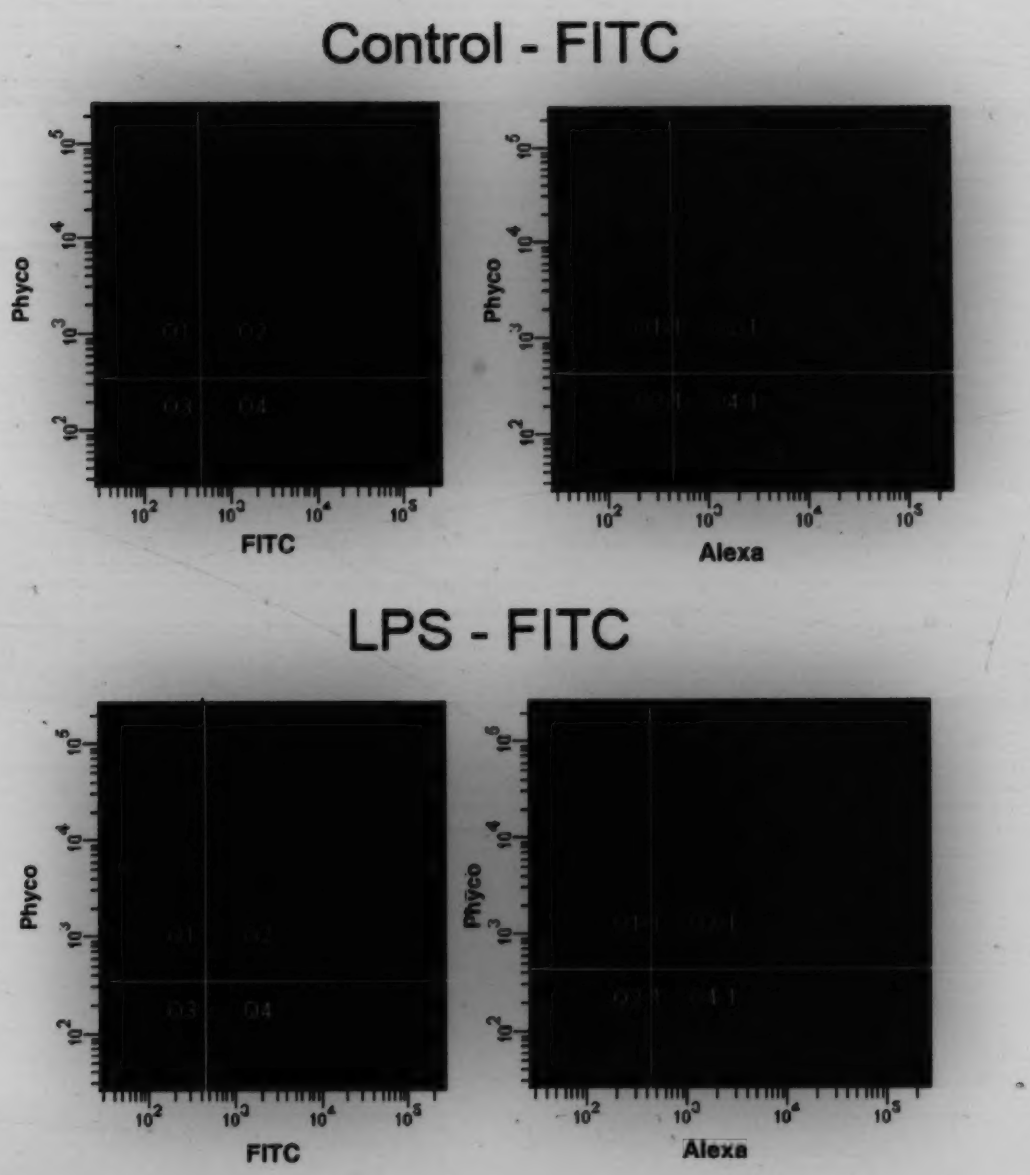


Figure A1.1 Two parameter dot plot histograms of staining by anti-annexin V-FITC (general marker of MPs) in the MP fraction of plasma from a control rat (Above, n = 1) and a LPS-treated rat (Below, n = 1). Left column: histogram of anti-CD62E-Phyco (Phyco) versus anti-annexin V-FITC (FITC). Right column: histogram of anti-CD62E-Phyco versus anti-CD31-Alexafluor 647 (Alexa).

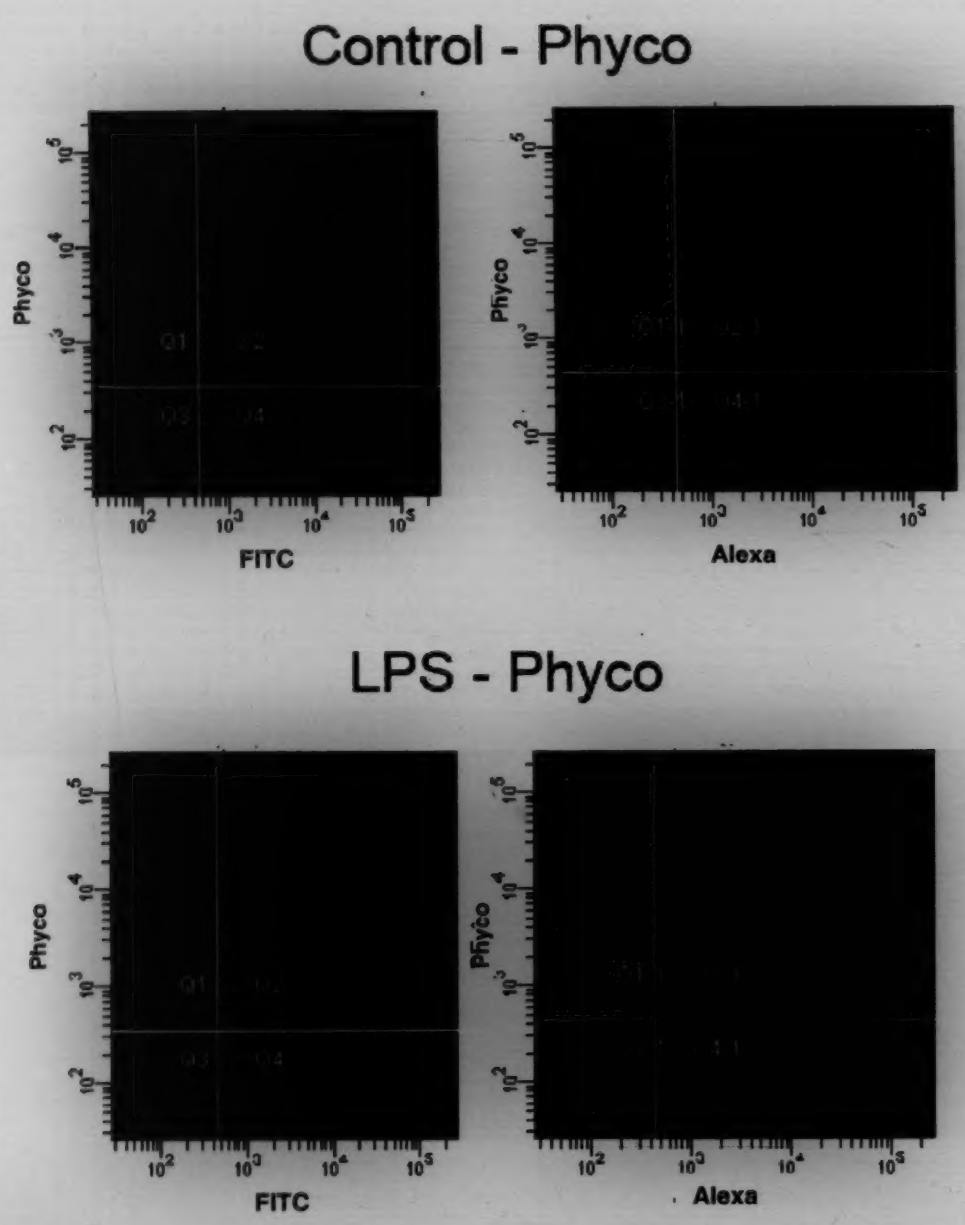


Figure A1.2 Two parameter dot plot histograms of staining by anti-CD62E-Phyco (marker of MPs from activated endothelial cells) in the MP fraction of plasma from a control rat (Above, n = 1) and a LPS-treated rat (Below, n = 1). Left column: histogram of anti-CD62E-Phyco (Phyco) versus anti-annexin V-FITC (FITC). Right column: histogram of anti-CD62E-Phyco versus anti-CD31-Alexafluor 647(Alexa).

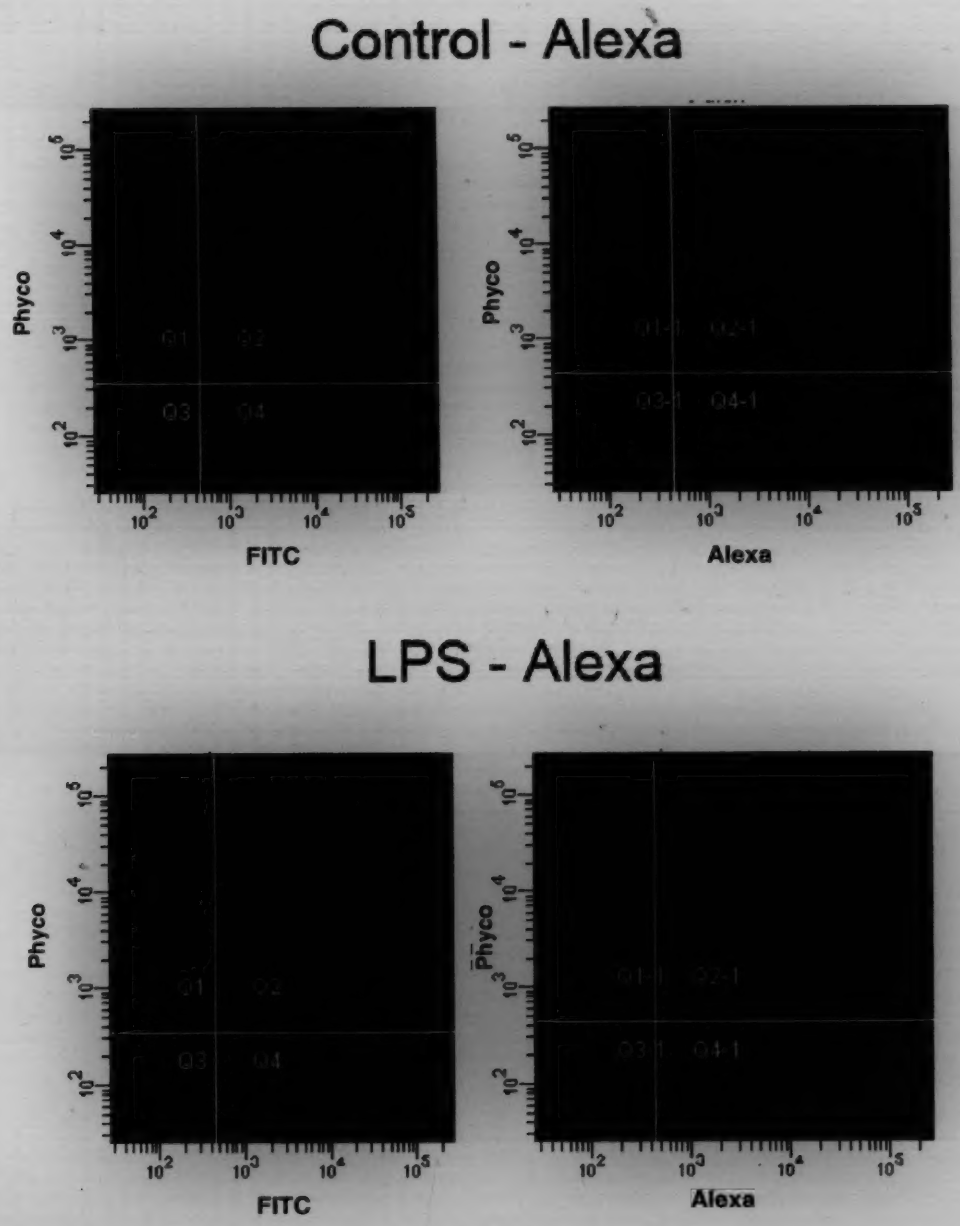


Figure A1.3 Two parameter dot plot histograms of staining by anti CD31-Alexafluor 647 (marker of MPs from apoptotic endothelial cells) in the MP fraction of plasma from a control rat (Above, n = 1) and a LPS-treated rat (Below, n = 1). Left column: histogram of anti-CD62E-Phyco (Phyco) versus anti-annexin V-FITC (FITC). Right column: histogram of anti-CD62E-Phyco versus anti-CD31-Alexafluor 647 (Alexa).

Table A1.1 Positive flow cytometric events of the microparticle fraction from the plasma of a control and a LPS-treated rat at 6 hr (Q, quadrant)

	FITC	PE	Alexa
Control	4234 (Q4)	19235 (Q1)	5354 (Q1-1)
LPS	15897 (Q4)	11401 (Q1)	23661 (Q1-1)

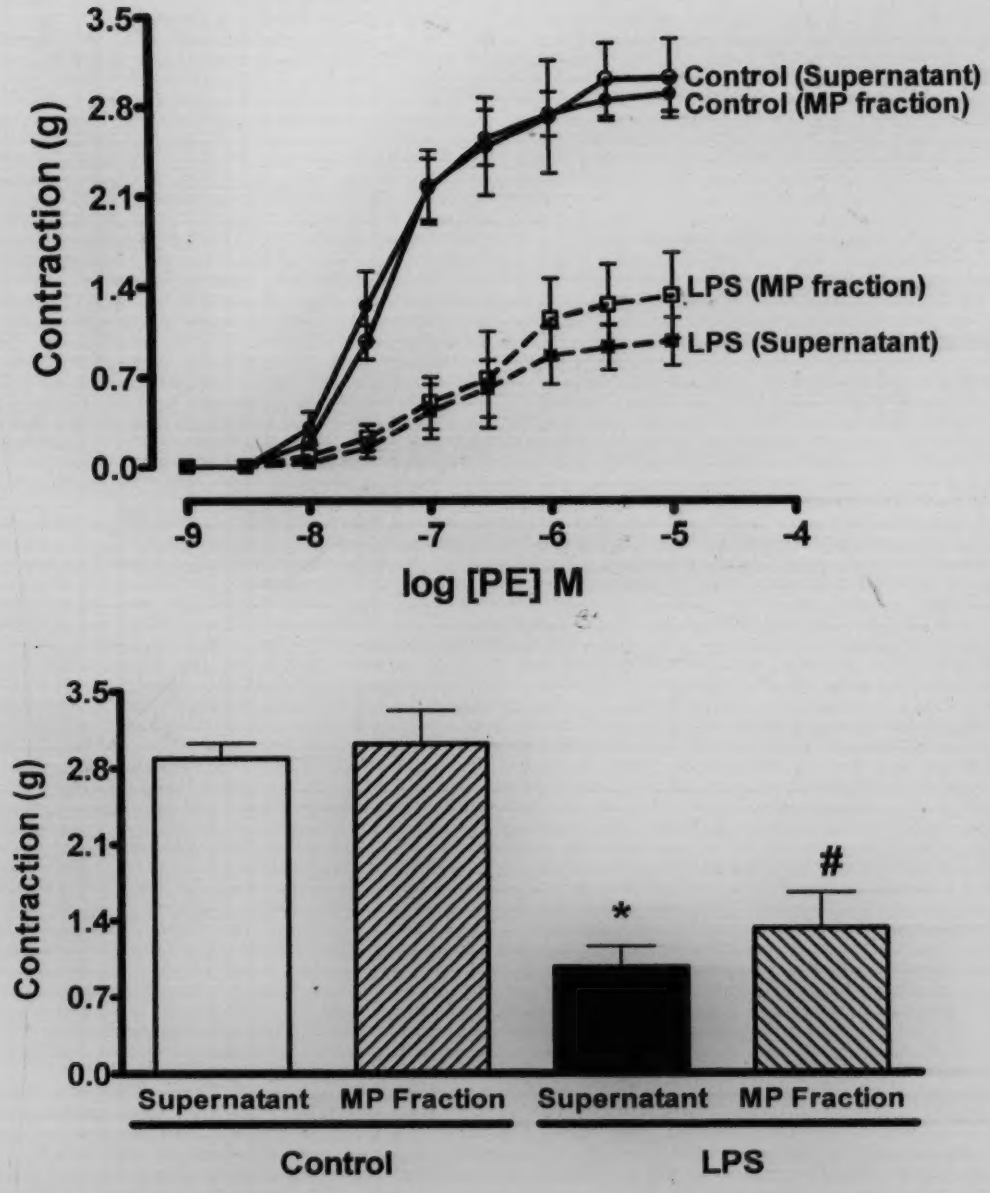


Figure A1.4 Above: concentration-response to PE of aortae incubated for 6 hr at 37 °C with Supernatant and MP fractions of the plasma from control and LPS-treated rats. Below: maximum contraction to PE (*$p < 0.05$ vs control supernatant, $^{\#}p < 0.05$ vs control MP fraction, one way ANOVA, Neuman-Keuls post hoc test, n = 4-5 aortic rings/group).

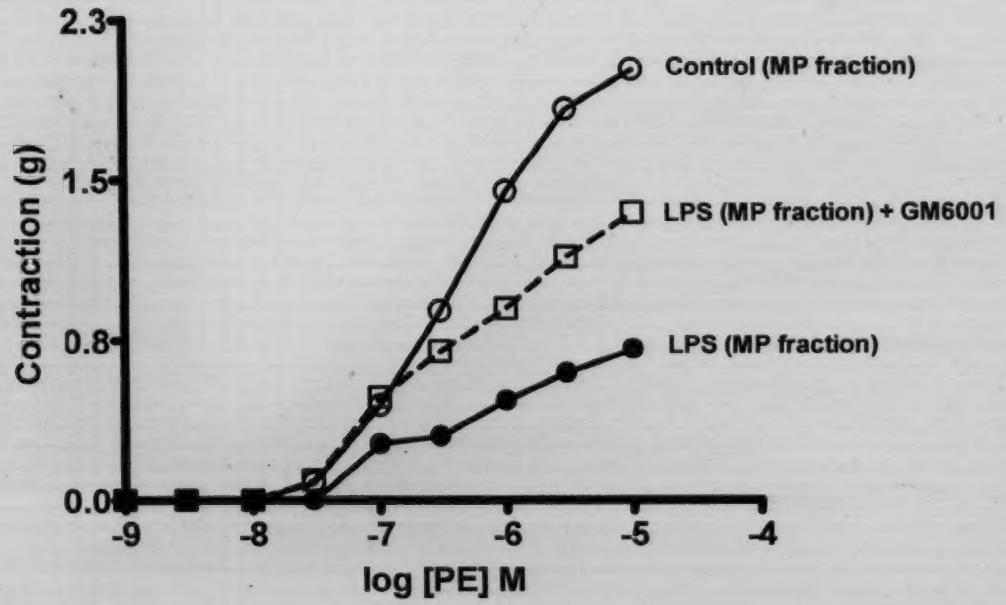


Figure A1.5 Concentration-response to PE from aortae incubated for 6 hr at 37 °C with microparticle (MP) fractions of plasma from control and LPS-treated rats in the presence or absence of GM6001 (30 μM). n = 2 aortic rings/group.

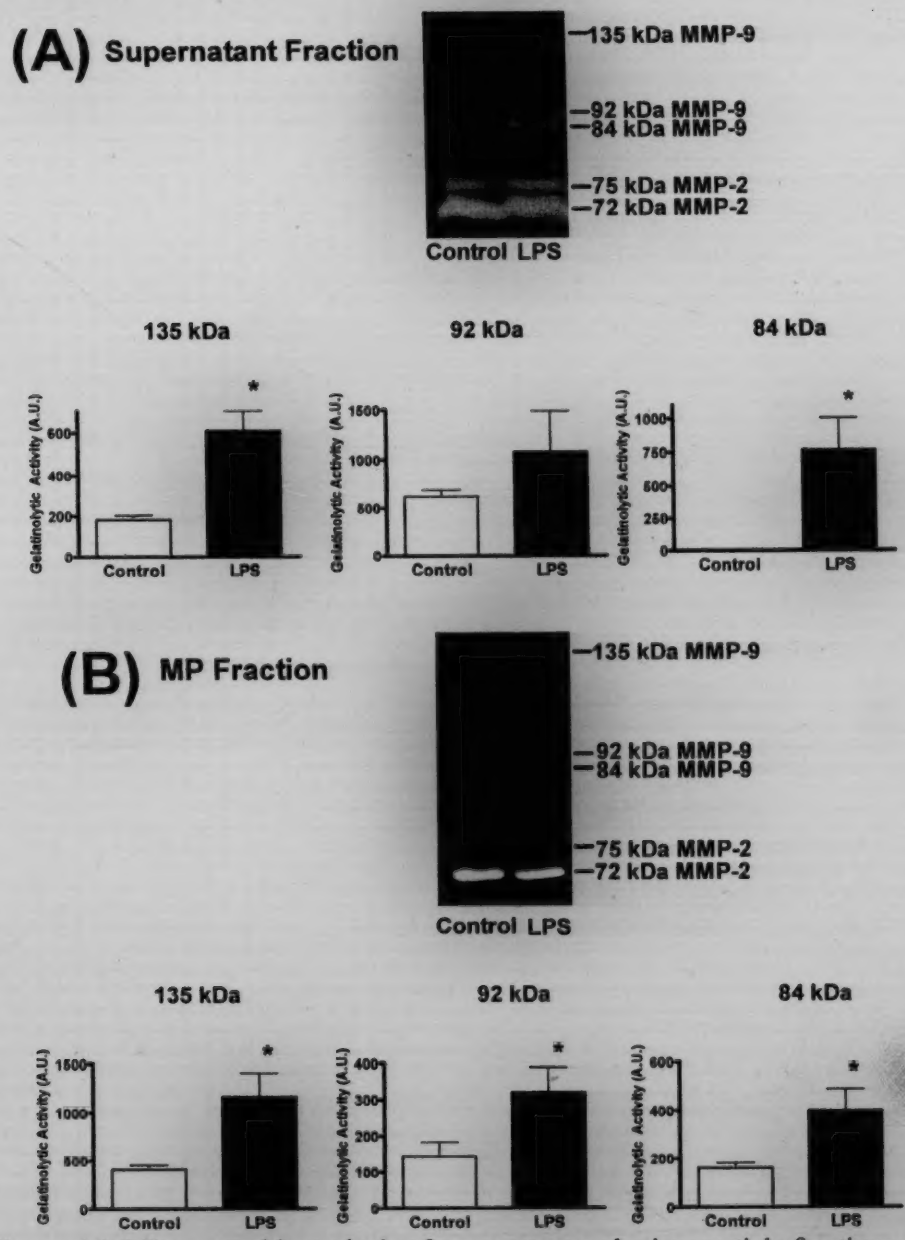


Figure A1.6 Zymographic analysis of supernatant and microparticle fractions of plasma from control and LPS-treated rats. (A) Top: representative zymogram of supernatant; Bottom-left: quantification of 135 kDa MMP-9 gelatinolytic activity; Bottom-middle: quantification of 92 kDa MMP-9 gelatinolytic activity; Bottom-right: quantification of 84 kDa MMP-9 gelatinolytic activity. (B) Top: representative zymogram of microparticle fraction; Bottom: quantification of MMP-9 gelatinolytic activity; Bottom-left: quantification of 135 kDa MMP-9 gelatinolytic activity; Bottom-middle: quantification of 92 kDa MMP-9 gelatinolytic activity; Bottom-right: quantification of 84 kDa MMP-9 gelatinolytic activity. *$p < 0.05$, unpaired t-test, n = 4 plasma samples/group.

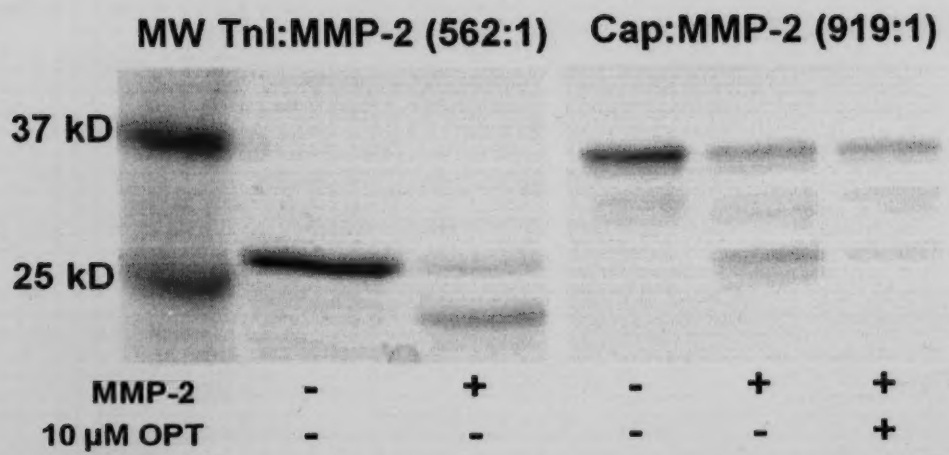


Figure A2.1 Coomassie blue staining of troponin I (TnI) and calponin (Cap) degradation by MMP-2 *in vitro*. Cap or TnI were incubated with human recombinant MMP-2 *in vitro* in the presence or absence of *o*-phenanthroline (OPT) for 2 hr at 37 ˚C. Left: MMP-2 mediated proteolysis of troponin I (TnI, 561:1 molar ratio); Right: MMP-2 mediated proteolysis of calponin (919:1 molar ratio).

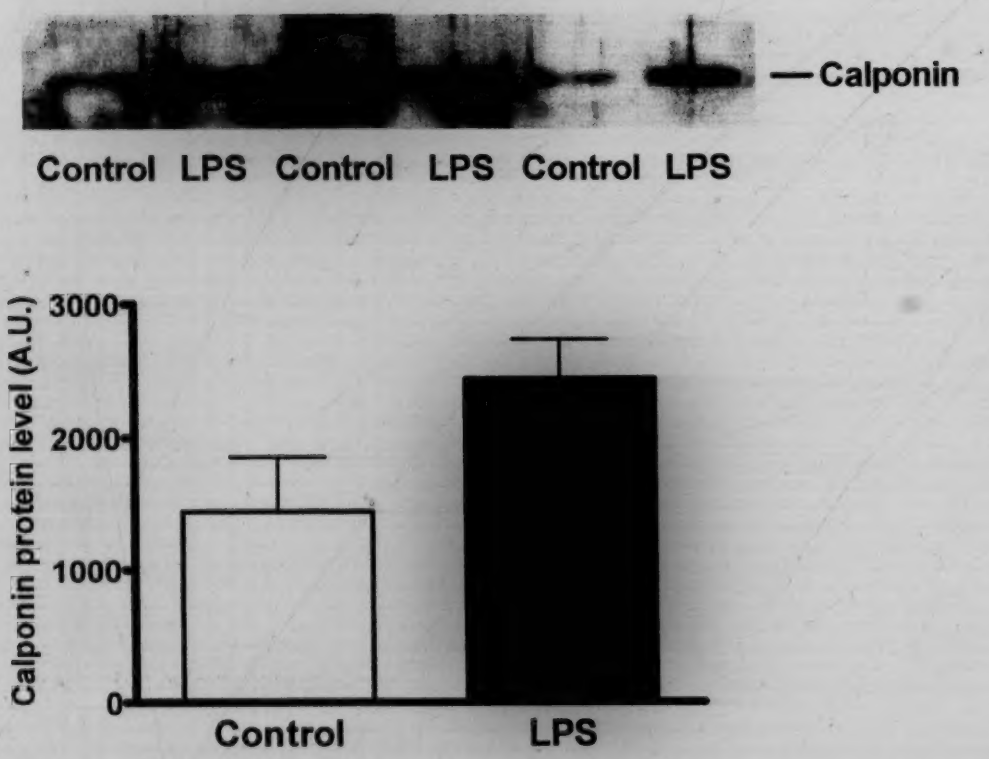


Figure A2.2 Calponin levels in aortic homogenates from control and LPS-treated rats. Top: representative immunoblot of calponin levels. Bottom: quantification of calponin levels (n = 4-5 aortic rings/group).

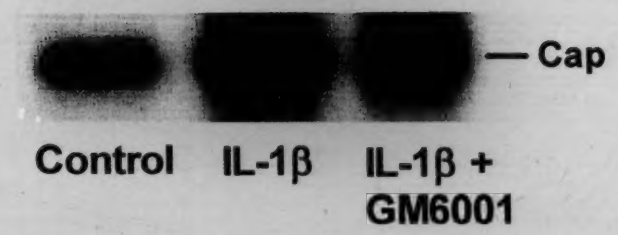


Figure A2.3 Calponin (Cap) levels in homogenates from aortae stimulated with IL-1β (10 ng/mL) for 6 hr at 37 °C in the presence or absence of GM6001 (30 μM). Immunoblot from one experiment (n = 1 pooled aorta/group).

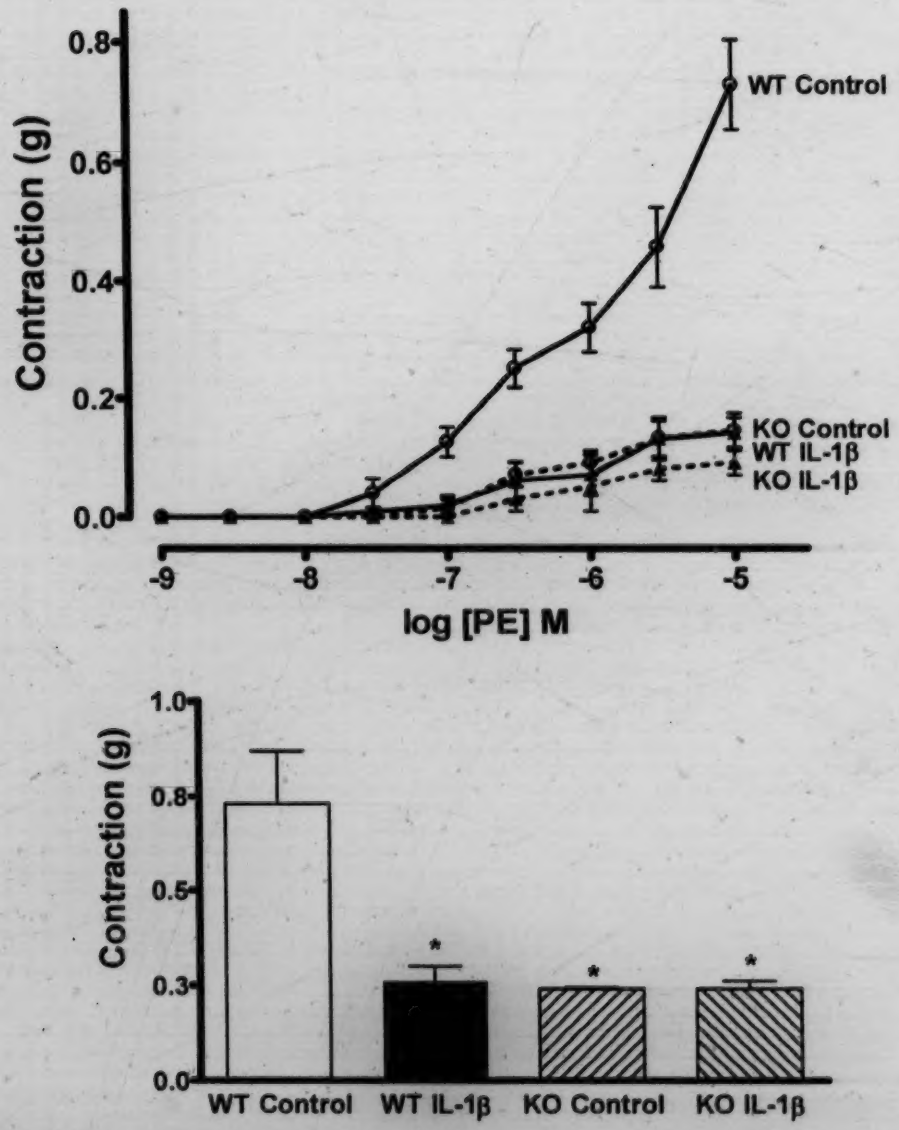


Figure A3.1 Above: concentration-response to PE in aortae from wild-type (WT) and MMP-2 knockout (KO) mice. Aortae incubated for 6 hr at 37 ˚C with IL-1β (10 ng/mL). Bottom: Quantification of 75 mM KCl response after washing. *$p < 0.05$ vs WT Control, one way ANOVA, Neuman-Keuls post hoc test, n = 3-4 aortic rings/group.

CHAPTER 8
REFERENCES

8.1 Reference list

1. Kumar A, Haery C, Parrillo JE: Myocardial dysfunction in septic shock: Part I. Clinical manifestation of cardiovascular dysfunction. *J Cardiothorac Vasc Anesth* 2001, 15:364-376.

2. Rivers EP, McIntyre L, Morro DC, Rivers KK: Early and innovative interventions for severe sepsis and septic shock: taking advantage of a window of opportunity. *Cmaj* 2005, 173:1054-1065.

3. Laupland K, Davies H, Church D, Louie T, Dool J, Zygun D, Doig C: Bloodstream Infection-Associated Sepsis and Septic Shock in Critically Ill Adults: A Population-Based Study. *Infection* 2004, 32: 59-64.

4. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G: 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* 2003, 31:1250-1256.

5. Bone RC: Modulators of coagulation. A critical appraisal of their role in sepsis. *Arch Intern Med* 1992, 152:1381-1389.

6. Marshall JC: Such stuff as dreams are made on: mediator-directed therapy in sepsis. *Nat Rev Drug Discov* 2003, 2:391-405.

7. Alexander C, Rietschel ET: Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 2001, 7:167-202.

8. Medzhitov R, Janeway C, Jr.: Innate immunity. *N Engl J Med* 2000, 343:338-344.

9. Antal-Szalmas P: Evaluation of CD14 in host defence. *Eur J Clin Invest* 2000, 30:167-179.

10. Landmann R, Muller B, Zimmerli W: CD14, new aspects of ligand and signal diversity. *Microbes Infect* 2000, 2:295-304.

11. Modlin RL, Brightbill HD, Godowski PJ: The toll of innate immunity on microbial pathogens. *N Engl J Med* 1999, 340:1834-1835.

12. Vasselon T, Detmers PA: Toll receptors: a central element in innate immune responses. *Infect Immun* 2002, 70:1033-1041.

13. Li X, Tupper JC, Bannerman DD, Winn RK, Rhodes CJ, Harlan JM: Phosphoinositide 3 kinase mediates Toll-like receptor 4-induced activation of NF-kappa B in endothelial cells. *Infect Immun* 2003, 71:4414-4420.

14. Wang JE, Dahle MK, McDonald M, Foster SJ, Aasen AO, Thiemermann C: Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock* 2003, 20:402-414.

15. Casey LC, Balk RA, Bone RC: Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann Intern Med* 1993, 119:771-778.

16. Endo S, Inada K, Yamada Y, Takakuwa T, Kasai T, Nakae H, Yoshida M, Ceska M: Plasma endotoxin and cytokine concentrations in patients with hemorrhagic shock. *Crit Care Med* 1994, 22:949-955.

17. Girardin E, Grau GE, Dayer JM, Roux-Lombard P, Lambert PH: Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N Engl J Med* 1988, 319:397-400.

18. Marchant A, Alegre ML, Hakim A, Pierard G, Marecaux G, Friedman G, De GD, Kahn RJ, Vincent JL, Goldman M: Clinical and biological significance of interleukin-10 plasma levels in patients with septic shock. *J Clin Immunol* 1995, 15:266-273.

19. Borrelli E, Roux-Lombard P, Grau GE, Girardin E, Ricou B, Dayer J, Suter PM: Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. *Crit Care Med* 1996, 24:392-397.

20. Natanson C, Danner RL, Fink MP, MacVittie TJ, Walker RI, Conklin JJ, Parrillo JE: Cardiovascular performance with E. coli challenges in a canine model of human sepsis. *Am J Physiol* 1988, 254:558-569.

21. Eichenholz PW, Eichacker PQ, Hoffman WD, Banks SM, Parrillo JE, Danner RL, Natanson C: Tumor necrosis factor challenges in canines: patterns of cardiovascular dysfunction. *Am J Physiol* 1992, 263:H668-H675.

22. Natanson C, Eichenholz PW, Danner RL, Eichacker PQ, Hoffman WD, Kuo GC, Banks SM, MacVittie TJ, Parrillo JE: Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *J Exp Med* 1989, 169:823-832.

23. Walley KR, Hebert PC, Wakai Y, Wilcox PG, Road JD, Cooper DJ: Decrease in left ventricular contractility after tumor necrosis factor-alpha infusion in dogs. *J Appl Physiol* 1994, 76:1060-1067.

24. Reinhart K, Karzai W: Anti-tumor necrosis factor therapy in sepsis: update on clinical trials and lessons learned. *Crit Care Med* 2001, 29:S121-S125.

25. Remick DG: Cytokine therapeutics for the treatment of sepsis: why has nothing worked? *Curr Pharm Des* 2003, 9:75-82.

26. Okusawa S, Gelfand JA, Ikejima T, Connolly RJ, Dinarello CA: Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *J Clin Invest* 1988, 81:1162-1172.

27. Gardiner SM, Kemp PA, March JE, Woolley J, Bennett T: The influence of antibodies to TNF-alpha and IL-1beta on haemodynamic responses to the cytokines, and to lipopolysaccharide, in conscious rats. *Br J Pharmacol* 1998, 125:1543-1550.

28. Ohlsson K, Bjork P, Bergenfeldt M, Hageman R, Thompson RC: Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature* 1990, 348:550-552.

29. Wakabayashi G, Gelfand JA, Burke JF, Thompson RC, Dinarello CA: A specific receptor antagonist for interleukin 1 prevents Escherichia coli-induced shock in rabbits. *Faseb J* 1991, 5:338-343.

30. Fisher CJ, Jr., Slotman GJ, Opal SM, Pribble JP, Bone RC, Emmanuel G, Ng D, Bloedow DC, Catalano MA: Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of sepsis syndrome: a randomized, open-label, placebo-controlled multicenter trial. *Crit Care Med* 1994, 22:12-21.

31. Echtenacher B, Weigl K, Lehn N, Mannel DN: Tumor necrosis factor-dependent adhesions as a major protective mechanism early in septic peritonitis in mice. *Infect Immun* 2001, 69:3550-3555.

32. Eskandari MK, Bolgos G, Miller C, Nguyen DT, DeForge LE, Remick DG: Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture or endotoxemia. *J Immunol* 1992, 148:2724-2730.

33. Opal SM: Severe sepsis and septic shock: defining the clinical problem. *Scand J Infect Dis* 2003, 35:529-534.

34. Riedemann NC, Guo RF, Ward PA: The enigma of sepsis. *J Clin Invest* 2003, 112:460-467.

35. Crowley SR: The pathogenesis of septic shock. *Heart Lung* 1996, 25:124-34.

36. Dasta JF: Norepinephrine in septic shock: renewed interest in an old drug. *DICP* 1990, 24:153-156.

37. Vila E, Salaices M: Cytokines and vascular reactivity in resistance arteries. *Am J Physiol Heart Circ Physiol* 2005, 288:1016-1021.

38. Macarthur H, Westfall TC, Riley DP, Misko TP, Salvemini D: Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. *Proc Natl Acad Sci USA* 2000, 97:9753-9758.

39. Chen SJ, Chen KH, Wu CC: Nitric oxide-cyclic GMP contributes to abnormal activation of Na+-K+-ATPase in the aorta from rats with endotoxic shock. *Shock* 2005, 23:179-185.

40. Sorrentino R, d'Emmanuele di Villa Bianca R, Lippolis L, Sorrentino L, Autore G, Pinto A: Involvement of ATP-sensitive potassium channels in a model of a delayed vascular hyporeactivity induced by lipopolysaccharide in rats. *Br J Pharmacol* 1999, 127:1447-1453.

41. Bassenge E: Endothelial function in different organs. *Prog Cardiovasc Dis* 1996, 39:209-228.

42. Grandel U, Grimminger F: Endothelial responses to bacterial toxins in sepsis. *Crit Rev Immunol* 2003, 23:267-299.

43. Vallet B: Bench-to-bedside review: endothelial cell dysfunction in severe sepsis: a role in organ dysfunction? *Crit Care* 2003, 7:130-138.

44. Grignani G, Maiolo A: Cytokines and hemostasis. *Haematologica* 2000, 85:967-972.

45. Rosenberg RD, Aird WC: Vascular-bed--specific hemostasis and hypercoagulable states. *N Engl J Med* 1999, 340:1555-1564.

46. Hernanz R, Alonso MJ, Zibrandtsen H, Alvarez Y, Salaices M, Simonsen U: Measurements of nitric oxide concentration and hyporeactivity in rat superior mesenteric artery exposed to endotoxin. *Cardiovasc Res* 2004, 62:202-211.

47. Stoclet JC, Martinez MC, Ohlmann P, Chasserot S, Schott C, Kleschyov AL, Schneider F, Andriantsitohaina R: Induction of nitric oxide synthase and dual effects of nitric oxide and cyclooxygenase products in regulation of arterial contraction in human septic shock. *Circulation* 1999, 100:107-112.

48. Miyamoto A, Moriki H, Ishiguro S, Nishio A: In vitro application of endotoxin enhances nitric oxide production in thoracic aortas from Mg-deficient rats. *Magnes Res* 2005, 18:155-162.

49. Miyamoto A, Moriki H, Ishiguro S, Nishio A: In vitro application of endotoxin to thoracic aortas from magnesium-deficient rats enhances vascular hyporeactivity to phenylephrine. *J Am Coll Nutr* 2004, 23:518S-520S.

50. Wright RC, Winkelmann RK: The epinephrine response of isolated rabbit vascular strips after in vivo and in vitro endotoxin exposure. *Angiology* 1971, 22:495-500.

51. Zingarelli B, Hasko G, Salzman AL, Szabo C: Effects of a novel guanylyl cyclase inhibitor on the vascular actions of nitric oxide and peroxynitrite in

immunostimulated smooth muscle cells and in endotoxic shock. *Crit Care Med* 1999, 27:1701-1707.

52. Martinez MC, Muller B, Stoclet JC, Andriantsitohaina R: Alteration by lipopolysaccharide of the relationship between intracellular calcium levels and contraction in rat mesenteric artery. *Br J Pharmacol* 1996, 118:1218-1222.

53. Luhm J, Kirchner H, Rink L: One-way synergistic effect of low superantigen concentrations on lipopolysaccharide-induced cytokine production. *J Interferon Cytokine Res* 1997, 17:229-238.

54. Clinton SK, Fleet JC, Loppnow H, Salomon RN, Clark BD, Cannon JG, Shaw AR, Dinarello CA, Libby P: Interleukin-1 gene expression in rabbit vascular tissue in vivo. *Am J Pathol* 1991, 138:1005-1014.

55. McKenna TM, Clegg JM, Williams TJ: Protein kinase C is a mediator of lipopolysaccharide-induced vascular suppression in the rat aorta. *Shock* 1994, 2:84-89.

56. O'Brien AJ, Thakur G, Buckley JF, Singer M, Clapp LH: The pore-forming subunit of the K(ATP) channel is an important molecular target for LPS-induced vascular hyporeactivity in vitro. *Br J Pharmacol* 2005, 144:367-375.

57. Wu CC, Chen SJ, Yen MH: Nitric oxide-independent activation of soluble guanylyl cyclase contributes to endotoxin shock in rats. *Am J Physiol* 1998, 275:1148-1157.

58. Chen SJ, Wu CC, Yang SN, Lin CI, Yen MH: Hyperpolarization contributes to vascular hyporeactivity in rats with lipopolysaccharide-induced endotoxic shock. *Life Sci* 2000, 68:659-668.

59. Chen SJ, Wu CC, Yang SN, Lin CI, Yen MH: Abnormal activation of K(+) channels in aortic smooth muscle of rats with endotoxic shock: electrophysiological and functional evidence. *Br J Pharmacol* 2000, 131:213-222.

60. Dorresteijn M, Smits P, van der HH, Pickkers P: Role of potassium channel blockade in the treatment of sepsis-induced vascular hyporeactivity. *Crit Care Med* 2006, 34:2867-2868.

61. Warrillow S, Egi M, Bellomo R: Randomized, double-blind, placebo-controlled crossover pilot study of a potassium channel blocker in patients with septic shock. *Crit Care Med* 2006, 34:980-985.

62. Sevransky J, Natanson C: Clinical trials in sepsis: an update. *Curr Opin Anaesthesiol* 2000, 13:125-129.

63. Freeman BD, Cobb JP: Nitric oxide synthase as a therapeutic target in sepsis--more questions than answers? *Crit Care Med* 1998, 26:1469-1470.

64. Cobb JP: Use of nitric oxide synthase inhibitors to treat septic shock: the light has changed from yellow to red. *Crit Care Med* 1999, 27:855-856.

65. Schulz R, Nava E, Moncada S: Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br J Pharmacol* 1992, 105:575-580.

66. Khadour FH, Panas D, Ferdinandy P, Schulze C, Csont T, Lalu MM, Wildhirt SM, Schulz R: Enhanced NO and superoxide generation in dysfunctional hearts from endotoxemic rats. *Am J Physiol Heart Circ Physiol* 2002, 283:1108-1115.

67. Suffredini AF, Fromm RE, Parker MM, Brenner M, Kovacs JA, Wesley RA, Parrillo JE: The cardiovascular response of normal humans to the administration of endotoxin. *N Engl J Med* 1989, 321:280-287.

68. Cunha FQ, Assreuy J, Moss DW, Rees D, Leal LM, Moncada S, Carrier M, O'Donnell CA, Liew FY: Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-alpha and IL-1-beta. *Immunology* 1994, 81:211-215.

69. d'Emmanuele di Villa Bianca R, Lippolis L, Autore G, Popolo A, Marzocco S, Sorrentino L, Pinto A, Sorrentino R: Dexamethasone improves vascular hyporeactivity induced by LPS in vivo by modulating ATP-sensitive potassium channels activity. *Br J Pharmacol* 2003, 140:91-96.

70. Hom GJ, Grant SK, Wolfe G, Bach TJ, MacIntyre DE, Hutchinson NI: Lipopolysaccharide-induced hypotension and vascular hyporeactivity in the rat: tissue analysis of nitric oxide synthase mRNA and protein expression in the

presence and absence of dexamethasone, NG-monomethyl-L-arginine or indomethacin. *J Pharmacol Exp Ther* 1995, 272:452-459.

71. Karimi G, Fatehi Z, Gholamnejad Z: The role of nitric oxide and protein kinase C in lipopolysaccharidemediated vascular hyporeactivity. *J Pharm Pharm Sci* 2006, 9:119-123.

72. Szabo C, Salzman AL, Ischiropoulos H: Endotoxin triggers the expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in the rat aorta in vivo. *FEBS Lett* 1995, 363:235-238.

73. Lalu MM, Gao CQ, Schulz R: Matrix metalloproteinase inhibitors attenuate endotoxemia induced cardiac dysfunction: a potential role for MMP-9. *Mol Cell Biochem* 2003, 251:61-66.

74. Lalu MM, Cena J, Chowdhury R, Lam A, Schulz R: Matrix metalloproteinases contribute to endotoxin and interleukin-1beta induced vascular dysfunction. *Br J Pharmacol* 2006, 149:31-42.

75. Lalu MM, Csont T, Schulz R: Matrix metalloproteinase activities are altered in the heart and plasma during endotoxemia. *Crit Care Med* 2004, 32:1332-1337.

76. Nava E, Palmer RM, Moncada S: The role of nitric oxide in endotoxic shock: effects of NG-monomethyl-L-arginine. *J Cardiovasc Pharmacol* 1992, 20 Suppl 12:132-134.

77. French JF, Lambert LE, Dage RC: Nitric oxide synthase inhibitors inhibit interleukin-1 beta-induced depression of vascular smooth muscle. *J Pharmacol Exp Ther* 1991, 259:260-264.

78. Wileman SM, Mann GE, Baydoun AR: Induction of L-arginine transport and nitric oxide synthase in vascular smooth muscle cells: synergistic actions of pro-inflammatory cytokines and bacterial lipopolysaccharide. *Br J Pharmacol* 1995, 116:3243-3250.

79. Wei CY, Huang KC, Chou YH, Hsieh PF, Lin KH, Lin WW: The role of Rho-associated kinase in differential regulation by statins of interleukin-1beta- and lipopolysaccharide-mediated nuclear factor kappaB activation and inducible nitric-oxide synthase gene expression in vascular smooth muscle cells. *Mol Pharmacol* 2006, 69:960-967.

80. Overall CM, McQuibban GA, Clark-Lewis I: Discovery of chemokine substrates for matrix metalloproteinases by exosite scanning: a new tool for degradomics. *Biol Chem* 2002, 383:1059-1066.

81. Schonbeck U, Mach F, Libby P: Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 1998, 161:3340-3346.

82. Shapira L, Soskolne WA, Houri Y, Barak V, Halabi A, Stabholz A: Protection against endotoxic shock and lipopolysaccharide-induced local inflammation by

tetracycline: correlation with inhibition of cytokine secretion. *Infect Immun* 1996, 64:825-828.

83. Ignarro LJ: Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension* 1990, 16:477-483.

84. Ignarro LJ: Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu Rev Pharmacol Toxicol* 1990, 30:535-560.

85. Moncada S, Palmer RM, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991, 43:109-142.

86. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980, 288:373-376.

87. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G: Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987, 84:9265-9269.

88. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987, 327:524-526.

89. Schulz R, Smith JA, Lewis MJ, Moncada S: Nitric oxide synthase in cultured endocardial cells of the pig. *Br J Pharmacol* 1991, 104:21-24.

90. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F: Purification and characterization of particulate endothelium-derived

relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci USA* 1991, 88:10480-10484.

91. Balligand JL, Kobzik L, Han X, Kaye DM, Belhassen L, O'Hara DS, Kelly RA, Smith TW, Michel T: Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (type III) nitric oxide synthase in cardiac myocytes. *J Biol Chem* 1995, 270:14582-14586.

92. Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC: Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc Natl Acad Sci USA* 1999, 96:657-662.

93. Calupca MA, Vizzard MA, Parsons RL: Origin of neuronal nitric oxide synthase (NOS)-immunoreactive fibers in guinea pig parasympathetic cardiac ganglia. *J Comp Neurol* 2000, 426:493-504.

94. Sawada K, Kondo T, Chang J, Inokuchi T, Aoyagi S: Distribution and neuropeptide content of nitric oxide synthase-containing nerve fibers in arteries and conduction system of the rat heart. *Acta Anat (Basel)* 1997, 160:239-247.

95. Toda N, Okamura T: The pharmacology of nitric oxide in the peripheral nervous system of blood vessels. *Pharmacol Rev* 2003, 55:271-324.

96. Radomski MW, Palmer RM, Moncada S: Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br J Pharmacol* 1987, 92:181-187.

97. Kubes P, Suzuki M, Granger DN: Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 1991, 88:4651-4655.

98. Radomski MW: Vascular endothelium in the processes of hemostasis and thrombosis. The role of prostacyclin and EDRF. *Acta Physiol Pol* 1989, 40 Suppl 33:97-109.

99. Knowles RG, Moncada S: Nitric oxide synthases in mammals. *Biochem J* 1994, 298 (Pt 2):249-258.

100. Murad F, Waldman S, Molina C, Bennett B, Leitman D: Regulation and role of guanylate cyclase-cyclic GMP in vascular relaxation. *Prog Clin Biol Res* 1987, 249:65-76.

101. Ignarro LJ: Haem-dependent activation of guanylate cyclase and cyclic GMP formation by endogenous nitric oxide: a unique transduction mechanism for transcellular signaling. *Pharmacol Toxicol* 1990, 67:1-7.

102. Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA: Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res* 2003, 93:280-291.

103. Munzel T, Feil R, Mulsch A, Lohmann SM, Hofmann F, Walter U: Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase. *Circulation* 2003, 108:2172-2183.

104. Birschmann I, Walter U: Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase. *Acta Biochim Pol* 2004, 51:397-404.

105. Smith JA, Radomski MW, Schulz R, Moncada S, Lewis MJ: Porcine ventricular endocardial cells in culture express the inducible form of nitric oxide synthase. *Br J Pharmacol* 1993, 108:1107-1010.

106. Radomski MW, Palmer RM, Moncada S: Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci USA* 1990, 87:10043-10047.

107. Balligand JL, Ungureanu-Longrois D, Simmons WW, Pimental D, Malinski TA, Kapturczak M, Taha Z, Lowenstein CJ, Davidoff AJ, Kelly RA *et al.*: Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro. *J Biol Chem* 1994, 269:27580-27588.

108. Rees DD, Cellek S, Palmer RM, Moncada S: Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem Biophys Res Commun* 1990, 173:541-547.

109. McCall TB, Palmer RM, Moncada S: Induction of nitric oxide synthase in rat peritoneal neutrophils and its inhibition by dexamethasone. *Eur J Immunol* 1991, 21:2523-2527.

110. Busse R, Mulsch A: Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett* 1990, 275:87-90.

111. Lirk P, Hoffmann G, Rieder J: Inducible nitric oxide synthase--time for reappraisal. *Curr Drug Targets Inflamm Allergy* 2002, 1:89-108.

112. Xia Y, Roman LJ, Masters BS, Zweier JL: Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J Biol Chem* 1998, 273:22635-22639.

113. Groeneveld PH, Kwappenberg KM, Langermans JA, Nibbering PH, Curtis L: Nitric oxide (NO) production correlates with renal insufficiency and multiple organ dysfunction syndrome in severe sepsis. *Intensive Care Med* 1996, 22:1197-1202.

114. Ochoa JB, Udekwu AO, Billiar TR, Curran RD, Cerra FB, Simmons RL, Peitzman AB: Nitrogen oxide levels in patients after trauma and during sepsis. *Ann Surg* 1991, 214:621-626.

115. Thiemermann C: Nitric oxide and septic shock. *Gen Pharmacol* 1997, 29:159-166.

116. Kilbourn RG, Jubran A, Gross SS, Griffith OW, Levi R, Adams J, Lodato RF: Reversal of endotoxin-mediated shock by NG-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem Biophys Res Commun* 1990, 172:1132-1138.

117. Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, Lodato RF: NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. *Proc Natl Acad Sci USA* 1990, 87:3629-3632.

118. Naseem KM: The role of nitric oxide in cardiovascular diseases. *Mol Aspects Med* 2005, 26:33-65.

119. Titheradge MA: Nitric oxide in septic shock. *Biochim Biophys Acta* 1999, 1411:437-455.

120. Whittle BJ: Nitric oxide in physiology and pathology. *Histochem J* 1995, 27:727-737.

121. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N *et al.*: Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995, 81:641-650.

122. Cobb JP: Nitric oxide synthase inhibition as therapy for sepsis: a decade of promise. *Surg Infect (Larchmt)* 2001, 2:93-100.

123. Cobb JP, Hotchkiss RS, Swanson PE, Chang K, Qiu Y, Laubach VE, Karl IE, Buchman TG: Inducible nitric oxide synthase (iNOS) gene deficiency increases the mortality of sepsis in mice. *Surgery* 1999, 126:438-442.

124. Nava E, Palmer RM, Moncada S: Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? *Lancet* 1991, 338:1555-1557.

125. Li T, Croce K, Winquist RJ: Regional differences in the effects of septic shock on vascular reactivity in the rabbit. *J Pharmacol Exp Ther* 1992, 261:959-963.

126. Rackow EC, Astiz ME: Pathophysiology and treatment of septic shock. *Jama* 1991, 266:548-554.

127. Schulz R, Panas DL, Catena R, Moncada S, Olley PM, Lopaschuk GD: The role of nitric oxide in cardiac depression induced by interleukin-1 beta and tumour necrosis factor-alpha. *Br J Pharmacol* 1995, 114:27-34.

128. McCord JM, Fridovich I: Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969, 244:6049-6055.

129. Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH, Jr. *et al.*: Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet* 1996, 13:43-47.

130. de Groot H: Reactive oxygen species in tissue injury. *Hepatogastroenterology* 1994, 41:328-332.

131. Kehrer JP: Free radicals as mediators of tissue injury and disease. *Crit Rev Toxicol* 1993, 23:21-48.

132. Brandes RP, Koddenberg G, Gwinner W, Kim D, Kruse HJ, Busse R, Mugge A: Role of increased production of superoxide anions by NAD(P)H oxidase and xanthine oxidase in prolonged endotoxemia. *Hypertension* 1999, 33:1243-1249.

133. Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R: Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res* 2000, 87:241-247.

134. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990, 87:1620-1624.

135. Kooy NW, Lewis SJ, Royall JA, Ye YZ, Kelly DR, Beckman JS: Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite. *Crit Care Med* 1997, 25:812-819.

136. Oyama J, Shimokawa H, Momii H, Cheng X, Fukuyama N, Arai Y, Egashira K, Nakazawa H, Takeshita A: Role of nitric oxide and peroxynitrite in the cytokine-induced sustained myocardial dysfunction in dogs in vivo. *J Clin Invest* 1998, 101:2207-2214.

137. Evgenov OV, Liaudet L: Role of nitrosative stress and activation of poly(ADP-ribose) polymerase-1 in cardiovascular failure associated with septic and hemorrhagic shock. *Curr Vasc Pharmacol* 2005, 3:293-299.

138. Tasatargil A, Dalaklioglu S, Sadan G: Inhibition of poly(ADP-ribose) polymerase prevents vascular hyporesponsiveness induced by lipopolysaccharide in isolated rat aorta. *Pharmacol Res* 2005, 51:581-586.

139. Pacher P, Schulz R, Liaudet L, Szabo C: Nitrosative stress and pharmacological modulation of heart failure. *Trends Pharmacol Sci* 2005, 26:302-310.

140. Mabley JG, Jagtap P, Perretti M, Getting SJ, Salzman AL, Virag L, Szabo E, Soriano FG, Liaudet L, Abdelkarim GE *et al.*: Anti-inflammatory effects of a novel, potent inhibitor of poly (ADP-ribose) polymerase. *Inflamm Res* 2001, 50:561-569.

141. Gross J, Lapiere CM: Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci USA* 1962, 48:1014-1022.

142. Van Wart HE, Birkedal-Hansen H: The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci USA* 1990, 87:5578-5582.

143. Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003, 92:827-839.

144. Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H: Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). *Biochemistry* 1990, 29:10261-10270.

145. Allan JA, Docherty AJ, Barker PJ, Huskisson NS, Reynolds JJ, Murphy G: Binding of gelatinases A and B to type-I collagen and other matrix components. *Biochem J* 1995, 309 (Pt 1):299-306.

146. Shipley JM, Wesselschmidt RL, Kobayashi DK, Ley TJ, Shapiro SD: Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc Natl Acad Sci USA* 1996, 93:3942-3946.

147. Li W, Gibson CW, Abrams WR, Andrews DW, DenBesten PK: Reduced hydrolysis of amelogenin may result in X-linked amelogenesis imperfecta. *Matrix Biol* 2001, 19:755-760.

148. Lohi J, Wilson CL, Roby JD, Parks WC: Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. *J Biol Chem* 2001, 276:10134-10144.

149. Morgunova E, Tuuttila A, Bergmann U, Isupov M, Lindqvist Y, Schneider G, Tryggvason K: Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. *Science* 1999, 284:1667-1670.

150. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI: Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 1995, 270:5331-5338.

151. Kang T, Nagase H, Pei D: Activation of membrane-type matrix metalloproteinase 3 zymogen by the proprotein convertase furin in the trans-Golgi network. *Cancer Res* 2002, 62:675-681.

152. Pei D, Weiss SJ: Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature* 1995, 375:244-247.

153. Sato H, Kinoshita T, Takino T, Nakayama K, Seiki M: Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. *FEBS Lett* 1996, 393:101-104.

154. Cao J, Rehemtulla A, Pavlaki M, Kozarekar P, Chiarelli C: Furin directly cleaves proMMP-2 in the trans-Golgi network resulting in a nonfunctioning proteinase. *J Biol Chem* 2005, 280:10974-10980.

155. Weiss SJ, Lampert MB, Test ST: Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science* 1983, 222:625-628.

156. Okamoto T, Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H: Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. *Arch Biochem Biophys* 1997, 342:261-274.

157. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS: Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of

vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996, 98:2572-2579.

158. Albrich JM, McCarthy CA, Hurst JK: Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. *Proc Natl Acad Sci USA* 1981, 78:210-214.

159. Galis ZS, Khatri JJ: Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res* 2002, 90:251-262.

160. Sawicki G, Salas E, Murat J, Miszta-Lane H, Radomski MW: Release of gelatinase A during platelet activation mediates aggregation. *Nature* 1997, 386:616-619.

161. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T: Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* 2003, 253:269-285.

162. Chow AK, Cena J, Schulz R: Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. *Br J Pharmacol* 2007. 152:189-205.

163. Schulz R: Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. *Annu Rev Pharmacol Toxicol* 2007, 47:211-242.

164. Brew K, Dinakarpandian D, Nagase H: Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 2000, 1477:267-283.

165. Li YY, McTiernan CF, Feldman AM: Proinflammatory cytokines regulate tissue inhibitors of metalloproteinases and disintegrin metalloproteinase in cardiac cells. *Cardiovasc Res* 1999, 42:162-172.

166. Martin EL, McCaig LA, Moyer BZ, Pape MC, Leco KJ, Lewis JF, Veldhuizen RA: Differential response of TIMP-3 null mice to the lung insults of sepsis, mechanical ventilation, and hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 2005, 289:244-251.

167. Leco KJ, Khokha R, Pavloff N, Hawkes SP, Edwards DR: Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J Biol Chem* 1994, 269:9352-9360.

168. Young DA, Phillips BW, Lundy C, Nuttall RK, Hogan A, Schultz GA, Leco KJ, Clark IM, Edwards DR: Identification of an initiator-like element essential for the expression of the tissue inhibitor of metalloproteinases-4 (Timp-4) gene. *Biochem J* 2002, 364:89-99.

169. Schulze CJ, Wang W, Suarez-Pinzon WL, Sawicka J, Sawicki G, Schulz R: Imbalance between tissue inhibitor of metalloproteinase-4 and matrix metalloproteinases during acute myocardial ischemia-reperfusion injury. *Circulation* 2003, 107:2487-2492.

170. Dollery CM, McEwan JR, Wang M, Sang QA, Liu YE, Shi YE: TIMP-4 is regulated by vascular injury in rats. *Ann N Y Acad Sci* 1999, 878:740-741.

171. Golub LM, Lee HM, Ryan ME, Giannobile WV, Payne J, Sorsa T: Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. *Adv Dent Res* 1998, 12:12-26.

172. Galardy RE, Cassabonne ME, Giese C, Gilbert JH, Lapierre F, Lopez H, Schaefer ME, Stack R, Sullivan M, Summers B *et al.*: Low molecular weight inhibitors in corneal ulceration. *Ann N Y Acad Sci* 1994, 732:315-323.

173. Liu Y, Ramamurthy N, Marecek J, Lee HM, Chen JL, Ryan ME, Rifkin BR, Golub LM: The lipophilicity, pharmacokinetics, and cellular uptake of different chemically-modified tetracyclines (CMTs). *Curr Med Chem* 2001, 8:243-252.

174. Golub LM, Ciancio S, Ramamamurthy NS, Leung M, McNamara TF: Low-dose doxycycline therapy: effect on gingival and crevicular fluid collagenase activity in humans. *J Periodontal Res* 1990, 25:321-330.

175. Garcia RA, Pantazatos DP, Gessner CR, Go KV, Woods VL, Jr., Villarreal FJ: Molecular interactions between matrilysin and the matrix metalloproteinase inhibitor doxycycline investigated by deuterium exchange mass spectrometry. *Mol Pharmacol* 2005, 67:1128-1136.

176. Peterson JT: The importance of estimating the therapeutic index in the development of matrix metalloproteinase inhibitors. *Cardiovasc Res* 2006, 69:677-687.

177. Fernandez-Patron C, Radomski MW, Davidge ST: Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res* 1999, 85:906-911.

178. Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW, Davidge ST: Vascular matrix metalloproteinase-2-dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. *Circ Res* 2000, 87:670-676.

179. Martinez A, Oh HR, Unsworth EJ, Bregonzio C, Saavedra JM, Stetler-Stevenson WG, Cuttitta F: Matrix metalloproteinase-2 cleavage of adrenomedullin produces a vasoconstrictor out of a vasodilator. *Biochem J* 2004, 383:413-418.

180. Chew DK, Conte MS, Khalil RA: Matrix metalloproteinase-specific inhibition of Ca2+ entry mechanisms of vascular contraction. *J Vasc Surg* 2004, 40:1001-1010.

181. Raffetto JD, Ross RL, Khalil RA: Matrix metalloproteinase 2-induced venous dilation via hyperpolarization and activation of K+ channels: relevance to varicose vein formation. *J Vasc Surg* 2007, 45:373-380.

182. Lipke DW, Soltis EE, Fiscus RR, Yang L, Newman PS, Aziz SM: RGD-containing peptides induce endothelium-dependent and independent vasorelaxations of rat aortic rings. *Regul Pept* 1996, 63:23-29.

183. Mogford JE, Davis GE, Platts SH, Meininger GA: Vascular smooth muscle alpha v beta 3 integrin mediates arteriolar vasodilation in response to RGD peptides. *Circ Res* 1996, 79:821-826.

184. D'Angelo G, Mogford JE, Davis GE, Davis MJ, Meininger GA: Integrin-mediated reduction in vascular smooth muscle [Ca2+]i induced by RGD-containing peptide. *Am J Physiol* 1997, 272:2065-2070.

185. Hamilton JR, Nguyen PB, Cocks TM: Atypical protease-activated receptor mediates endothelium-dependent relaxation of human coronary arteries. *Circ Res* 1998, 82:1306-1311.

186. Mandal M, Das S, Chakraborti T, Mandal A, Chakraborti S: Role of matrix metalloprotease-2 in oxidant activation of Ca2+ ATPase by hydrogen peroxide in pulmonary vascular smooth muscle plasma membrane. *J Biosci* 2003, 28:205-213.

187. Das S, Chakraborti T, Mandal M, Mandal A, Chakraborti S: Role of membrane-associated Ca+ dependent matrix metalloprotease-2 in the oxidant activation of Ca2+Atpase by tertiary butylhydroperoxide. *Mol Cell Biochem* 2002, 237:85-93.

188. Jeyabalan A, Novak J, Doty KD, Matthews J, Fisher MC, Kerchner LJ, Conrad KP: Vascular matrix metalloproteinase-9 mediates the inhibition of myogenic reactivity in small arteries isolated from rats after short-term administration of relaxin. *Endocrinology* 2007, 148:189-197.

189. Hao L, Du M, Lopez-Campistrous A, Fernandez-Patron C: Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway. *Circ Res* 2004, 94:68-76.

190. Hao L, Nishimura T, Wo H, Fernandez-Patron C: Vascular responses to alpha1-adrenergic receptors in small rat mesenteric arteries depend on mitochondrial reactive oxygen species. *Arterioscler Thromb Vasc Biol* 2006, 26:819-825.

191. He JZ, Quan A, Xu Y, Teoh H, Wang G, Fish JE, Steer BM, Itohara S, Marsden PA, Davidge ST *et al.*: Induction of matrix metalloproteinase-2 enhances systemic arterial contraction after hypoxia. *Am J Physiol Heart Circ Physiol* 2007, 292:H684-H693.

192. Xie B, Dong Z, Fidler IJ: Regulatory mechanisms for the expression of type IV collagenases/gelatinases in murine macrophages. *J Immunol* 1994, 152:3637-3644.

193. Pugin J, Widmer MC, Kossodo S, Liang CM, Preas HLn, Suffredini AF: Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. *Am J Respir Cell Mol Biol* 1999, 20:458-464.

194. Albert J, Radomski A, Soop A, Sollevi A, Frostell C, Radomski MW: Differential release of matrix metalloproteinase-9 and nitric oxide following infusion of endotoxin to human volunteers. *Acta Anaesthesiol Scand* 2003, 47:407-410.

195. Paemen L, Jansen PM, Proost P, Van Damme J, Opdenakker G, Hack E, Taylor FB: Induction of gelatinase B and MCP-2 in baboons during sublethal and lethal bacteraemia. *Cytokine* 1997, 9:412-415.

196. Dubois B, Starckx S, Pagenstecher A, Oord J, Arnold B, Opdenakker G: Gelatinase B deficiency protects against endotoxin shock. *Eur J Immunol* 2002, 32:2163-2171.

197. Carney DE, McCann UG, Schiller HJ, Gatto LA, Steinberg J, Picone AL, Nieman GF: Metalloproteinase inhibition prevents acute respiratory distress syndrome. *J Surg Res* 2001, 99:245-252.

198. Steinberg J, Halter J, Schiller H, Gatto L, Carney D, Lee HM, Golub L, Nieman G: Chemically modified tetracycline prevents the development of septic shock and acute respiratory distress syndrome in a clinically applicable porcine model. *Shock* 2005, 24:348-356.

199. Martin EL, Moyer BZ, Pape MC, Starcher B, Leco KJ, Veldhuizen RA: Negative impact of tissue inhibitor of metalloproteinase-3 null mutation on lung structure and function in response to sepsis. *Am J Physiol Lung Cell Mol Physiol* 2003, 285:1222-1232.

200. Gao CQ, Sawicki G, Suarez-Pinzon WL, Csont T, Wozniak M, Ferdinandy P, Schulz R: Matrix metalloproteinase-2 mediates cytokine-induced myocardial contractile dysfunction. *Cardiovasc Res* 2003, 57:426-433.

201. Zhang K, McQuibban GA, Silva C, Butler GS, Johnston JB, Holden J, Clark-Lewis I, Overall CM, Power C: HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. *Nat Neurosci* 2003, 6:1064-1071.

202. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM: Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 2002, 100:1160-1167.

203. Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V: Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Pathol* 2002, 160:673-680.

204. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R: Microparticles in cardiovascular diseases. *Cardiovasc Res* 2003, 59:277-287.

205. Aras O, Shet A, Bach RR, Hysjulien JL, Slungaard A, Hebbel RP, Escolar G, Jilma B, Key NS: Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. *Blood* 2004, 103:4545-4553.

206. Meziani F, Tesse A, David E, Martinez MC, Wangesteen R, Schneider F, Andriantsitohaina R: Shed membrane particles from preeclamptic women generate vascular wall inflammation and blunt vascular contractility. *Am J Pathol* 2006, 169:1473-1483.

207. Nieuwland R, Berckmans RJ, McGregor S, Boing AN, Romijn FP, Westendorp RG, Hack CE, Sturk A: Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 2000, 95:930-935.

208. Soriano AO, Jy W, Chirinos JA, Valdivia MA, Velasquez HS, Jimenez JJ, Horstman LL, Kett DH, Schein RM, Ahn YS: Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis. *Crit Care Med* 2005, 33:2540-2546.

209. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R: Microparticles in cardiovascular diseases. *Cardiovasc Res* 2003, 59:277-287.

210. Tesse A, Martinez MC, Hugel B, Chalupsky K, Muller CD, Meziani F, Mitolo-Chieppa D, Freyssinet JM, Andriantsitohaina R: Upregulation of proinflammatory proteins through NF-kappaB pathway by shed membrane microparticles results in vascular hyporeactivity. *Arterioscler Thromb Vasc Biol* 2005, 25:2522-2527.

211. VanWijk MJ, Nieuwland R, Boer K, van der Post JA, VanBavel E, Sturk A: Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction? *Am J Obstet Gynecol* 2002, 187:450-456.

212. VanWijk MJ, Svedas E, Boer K, Nieuwland R, VanBavel E, Kublickiene KR: Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from healthy pregnant women. *Am J Obstet Gynecol* 2002, 187:1686-1693.

213. Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JR, Sawicki G, Schulz R: Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 2002, 106:1543-1549.

214. Takahashi K, Nadal-Ginard B: Molecular cloning and sequence analysis of smooth muscle calponin. *J Biol Chem* 1991, 266:13284-13288.

215. North AJ, Gimona M, Cross RA, Small JV: Calponin is localised in both the contractile apparatus and the cytoskeleton of smooth muscle cells. *J Cell Sci* 1994, 107 (Pt 3):437-444.

216. Tsunekawa S, Takahashi K, Abe M, Hiwada K, Ozawa K, Murachi T: Calpain proteolysis of free and bound forms of calponin, a troponin T-like protein in smooth muscle. *FEBS Lett* 1989, 250:493-496.

217. Maekawa A, Lee JK, Nagaya T, Kamiya K, Yasui K, Horiba M, Miwa K, Uzzaman M, Maki M, Ueda Y *et al.*: Overexpression of calpastatin by gene transfer prevents troponin I degradation and ameliorates contractile dysfunction in rat hearts subjected to ischemia/reperfusion. *J Mol Cell Cardiol* 2003, 35:1277-1284.

218. Yamada A, Uegaki A, Nakamura T, Ogawa K: ONO-4817, an orally active matrix metalloproteinase inhibitor, prevents lipopolysaccharide-induced proteoglycan release from the joint cartilage in guinea pigs. *Inflamm Res* 2000, 49:144-146.

219. Nunes JP: Effects of lipopolysaccharide on vascular reactivity and mortality in rats. *Auton Autacoid Pharmacol* 2002, 22:247-252.

220. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R: Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000, 101:1833-1839.

221. Heussen C, Dowdle EB: Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* 1980, 102:196-202.

222. Spinale FG: Matrix metalloproteinases: regulation and dysregulation in the failing heart. *Circ Res* 2002, 90:520-530.

223. Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, Szczesna-Cordary D, Schulz R: Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. *Circulation* 2005, 112:544-552.

224. Chew DK, Conte MS, Khalil RA: Matrix metalloproteinase-specific inhibition of Ca2+ entry mechanisms of vascular contraction. *J Vasc Surg* 2004, 40:1001-1010.

225. Fernandez-Patron C, Radomski MW, Davidge ST: Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res* 1999, 85:906-911.

226. Jeyabalan A, Novak J, Danielson LA, Kerchner LJ, Opett SL, Conrad KP: Essential role for vascular gelatinase activity in relaxin-induced renal vasodilation, hyperfiltration, and reduced myogenic reactivity of small arteries. *Circ Res* 2003, 93:1249-1257.

227. McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM: Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 2000, 289:1202-1206.

228. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR: Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001, 29:1303-1310.

229. Rees DD, Monkhouse JE, Cambridge D, Moncada S: Nitric oxide and the haemodynamic profile of endotoxin shock in the conscious mouse. *Br J Pharmacol* 1998, 124:540-546.

230. Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino MA, Jr., Cerami A, Shires GT, Lowry SF: Cytokine appearance in human endotoxemia and primate bacteremia. *Surg Gynecol Obstet* 1988, 166:147-153.

231. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A: Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* 1987, 330:662-664.

232. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ, III, Zentella A, Albert JD *et al.*: Shock and tissue injury induced by recombinant human cachectin. *Science* 1986, 234:470-474.

233. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996, 271:C1424-C1437.

234. Javesghani D HSSJQMMS: Superoxide production in the vasculature of lipopolysaccharide-treated rats and pigs. *Shock* 2003, 19:486-493.

235. Moreno JJ, Pryor WA: Inactivation of alpha 1-proteinase inhibitor by peroxynitrite. *Chem Res Toxicol* 1992, 5:425-431.

236. Radi R, Beckman JS, Bush KM, Freeman BA: Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991, 288:481-487.

237. Okamoto T, Akaike T, Sawa T, Miyamoto Y, van der Vliet A, Maeda H: Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *J Biol Chem* 2001, 276:29596-29602.

238. Frears ER, Zhang Z, Blake DR, O'Connell JP, Winyard PG: Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Lett* 1996, 381:21-24.

239. Kim H, Koh G: Lipopolysaccharide activates matrix metalloproteinase-2 in endothelial cells through an NF-kappaB-dependent pathway. *Biochem Biophys Res Commun* 2000, 269:401-405.

240. Nakamura T, Ebihara I, Shimada N, Shoji H, Koide H: Modulation of plasma metalloproteinase-9 concentrations and peripheral blood monocyte mRNA levels in patients with septic shock: effect of fiber-immobilized polymyxin B treatment. *Am J Med Sci* 1998, 316:355-360.

241. Pagenstecher A, Stalder AK, Kincaid CL, Volk B, Campbell IL: Regulation of matrix metalloproteinases and their inhibitor genes in lipopolysaccharide-induced endotoxemia in mice. *Am J Pathol* 2000, 157:197-210.

242. Danner RL, Joiner KA, Rubin M, Patterson WH, Johnson N, Ayers KM, Parrillo JE: Purification, toxicity, and antiendotoxin activity of polymyxin B nonapeptide. *Antimicrob Agents Chemother* 1989, 33:1428-1434.

243. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982, 126:131-138.

244. Sang QX, Stetler-Stevenson WG, Liotta LA, Byers SW: Identification of type IV collagenase in rat testicular cell culture: influence of peritubular-Sertoli cell interactions. *Biol Reprod* 1990, 43:956-964.

245. Gui Y, Zheng XL, Hollenberg MD: Interleukin-1beta, Src- and non-Src tyrosine kinases, and nitric oxide synthase induction in rat aorta in vitro. *Am J Physiol Heart Circ Physiol* 2000, 279:H566-H576.

246. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P: Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res* 1994, 75:181-189.

247. Hu J, Van den Steen PE, Dillen C, Opdenakker G: Targeting neutrophil collagenase/matrix metalloproteinase-8 and gelatinase B/matrix

metalloproteinase-9 with a peptidomimetic inhibitor protects against endotoxin shock. *Biochem Pharmacol* 2005, 70:535-544.

248. Haug C, Lenz C, Diaz F, Bachem MG: Oxidized low-density lipoproteins stimulate extracellular matrix metalloproteinase Inducer (EMMPRIN) release by coronary smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004, 24:1823-1829.

249. Gurjar MV, Deleon J, Sharma RV, Bhalla RC: Role of reactive oxygen species in IL-1 beta-stimulated sustained ERK activation and MMP-9 induction. *Am J Physiol Heart Circ Physiol* 2001, 281:H2568-H2574.

250. Rajavashisth TB, Liao JK, Galis ZS, Tripathi S, Laufs U, Tripathi J, Chai NN, Xu XP, Jovinge S, Shah PK *et al.*: Inflammatory cytokines and oxidized low density lipoproteins increase endothelial cell expression of membrane type 1-matrix metalloproteinase. *J Biol Chem* 1999, 274:11924-11929.

251. Mandal M, Mandal A, Das S, Chakraborti T, Sajal C: Clinical implications of matrix metalloproteinases. *Mol Cell Biochem* 2003, 252:305-329.

252. Galley HF, Webster NR: Physiology of the endothelium. *Br J Anaesth* 2004, 93:105-113.

253. Boos CJ, Goon PK, Lip GY: The endothelium, inflammation, and coagulation in sepsis. *Clin Pharmacol Ther* 2006, 79:20-2.

254. Hotchkiss RS, Karl IE: Endothelial cell apoptosis in sepsis: a case of habeas corpus? *Crit Care Med* 2004, 32:901-902.

255. Julou-Schaeffer G, Gray GA, Fleming I, Schott C, Parratt JR, Stoclet JC: Loss of vascular responsiveness induced by endotoxin involves L-arginine pathway. *Am J Physiol* 1990, 259:1038-1043.

256. Nakaki T, Otsuka Y, Nakayama M, Kato R: Endothelium-accelerated hyporesponsiveness of norepinephrine-elicited contraction of rat aorta in the presence of bacterial lipopolysaccharide. *Eur J Pharmacol* 1992, 219:311-318.

257. Fleming I, Gray GA, Stoclet JC: Influence of endothelium on induction of the L-arginine-nitric oxide pathway in rat aortas. *Am J Physiol* 1993, 264:1200-1207.

258. van Beusekom HM, Post MJ, Whelan DM, de Smet BJ, Duncker DJ, van der Giessen WJ: Metalloproteinase inhibition by batimastat does not reduce neointimal thickening in stented atherosclerotic porcine femoral arteries. *Cardiovasc Radiat Med* 2003, 4:186-191.

259. Buckley JF, Singer M, Clapp LH: Role of K(ATP) channels in sepsis. *Cardiovasc Res* 2006, 144: 367-375.

260. Puyraimond A, Fridman R, Lemesle M, Arbeille B, Menashi S: MMP-2 colocalizes with caveolae on the surface of endothelial cells. *Exp Cell Res* 2001, 262:28-36.

261. Lei MG, Morrison DC: Differential expression of caveolin-1 in lipopolysaccharide-activated murine macrophages. *Infect Immun* 2000, 68:5084-5089.

262. Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW, Davidge ST: Vascular matrix metalloproteinase-2-dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. *Circ Res* 2000, 87:670-676.

263. Sung MM, Schulz CG, Wang W, Sawicki G, Bautista-Lopez NL, Schulz R: Matrix metalloproteinase-2 degrades the cytoskeletal protein alpha-actinin in peroxynitrite mediated myocardial injury. *J Mol Cell Cardiol* 2007, 43:429-436.

264. Pastor CM, Billiar TR: Nitric oxide causes hyporeactivity to phenylephrine in isolated perfused livers from endotoxin-treated rats. *Am J Physiol* 1995, 268:G177-G182.

265. Fleming I, Gray GA, Julou-Schaeffer G, Parratt JR, Stoclet JC: Incubation with endotoxin activates the L-arginine pathway in vascular tissue. *Biochem Biophys Res Commun* 1990, 171:562-568.

266. Alvarez B, Radi R: Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* 2003, 25:295-311.

267. Chow AK, Cena J, Schulz R: Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. *Br J Pharmacol* 2007, 152:189-205.

268. Schulz R: Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. *Annu Rev Pharmacol Toxicol* 2007, 47:211-242.

269. Lehti K, Lohi J, Valtanen H, Keski-Oja J: Proteolytic processing of membrane-type-1 matrix metalloproteinase is associated with gelatinase A activation at the cell surface. *Biochem J* 1998, 334 (Pt 2):345-353.

270. Szabo C, Zingarelli B, Salzman AL: Role of poly-ADP ribosyltransferase activation in the vascular contractile and energetic failure elicited by exogenous and endogenous nitric oxide and peroxynitrite. *Circ Res* 1996, 78:1051-1063.

271. Pagenstecher A, Stalder AK, Campbell IL: RNAse protection assays for the simultaneous and semiquantitative analysis of multiple murine matrix metalloproteinase (MMP) and MMP inhibitor mRNAs. *J Immunol Methods* 1997, 206:1-9.

272. Pagenstecher A, Stalder AK, Kincaid CL, Volk B, Campbell IL: Regulation of matrix metalloproteinases and their inhibitor genes in lipopolysaccharide-induced endotoxemia in mice. *Am J Pathol* 2000, 157:197-210.

273. Liu P, Sun M, Sader S: Matrix metalloproteinases in cardiovascular disease. *Can J Cardiol* 2006, 22 Suppl B:25B-30B.

274. Hidalgo M, Eckhardt SG: Development of matrix metalloproteinase inhibitors in cancer therapy. *J Natl Cancer Inst* 2001, 93:178-193.

275. Amin AR, Attur MG, Thakker GD, Patel PD, Vyas PR, Patel RN, Patel IR, Abramson SB: A novel mechanism of action of tetracyclines: effects on nitric oxide synthases. *Proc Natl Acad Sci USA* 1996, 93:14014-14019.

276. Sadowski T, Steinmeyer J: Minocycline inhibits the production of inducible nitric oxide synthase in articular chondrocytes. *J Rheumatol* 2001, 28:336-340.

277. Maitra SR, Bhaduri S, Valane PD, Tervahartiala T, Sorsa T, Ramamurthy N: Inhibition of matrix metalloproteinases by chemically modified tetracyclines in sepsis. *Shock* 2003, 20:280-285.

278. Kishnani NS, Tabrizi-Fard MA, Fung HL: Diethyldithiocarbamate prolongs survival of mice in a lipopolysaccharide-induced endotoxic shock model: evidence for multiple mechanisms. *Shock* 1999, 11:264-268.

279. Marcet-Palacios M, Graham K, Cass C, Befus AD, Mayers I, Radomski MW: Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. *J Pharmacol Exp Ther* 2003, 307:429-436.

280. Masure S, Proost P, Van Damme J, Opdenakker G: Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *Eur J Biochem* 1991, 198:391-398.

281. Opdenakker G, Masure S, Grillet B, Van Damme J: Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis. *Lymphokine Cytokine Res* 1991, 10:317-324.

282. Cena J, Lalu MM, Rosenfelt C, Schulz R: Endothelial dependence of matrix metalloproteinase-mediated vascular hyporeactivity caused by lipopolysaccharide. *Eur J Pharmacol* 2007, 586:116-122.

283. ter Steege JC, van de Ven MW, Forget PP, Brouckaert P, Buurman WA: The role of endogenous IFN-gamma, TNF-alpha and IL-10 in LPS-induced nitric oxide release in a mouse model. *Cytokine* 1998, 10:115-123.

284. Dinarello CA, Okusawa S, Gelfand JA: Interleukin-1 induces a shock-like state in rabbits: synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *Prog Clin Biol Res* 1989, 286:243-263.

285. Gui Y, Zheng XL, Hollenberg MD: Interleukin-1beta, Src- and non-Src tyrosine kinases, and nitric oxide synthase induction in rat aorta in vitro. *Am J Physiol Heart Circ Physiol* 2000, 279:H566-H576.

286. Balligand JL, Ungureanu-Longrois D, Simmons WW, Kobzik L, Lowenstein CJ, Lamas S, Kelly RA, Smith TW, Michel T: Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1 beta and IFN-gamma. *Am J Physiol* 1995, 268:1293-303.

287. Wakabayashi G, Gelfand JA, Burke JF, Thompson RC, Dinarello CA: A specific receptor antagonist for interleukin 1 prevents Escherichia coli-induced shock in rabbits. *Faseb J* 1991, 5:338-343.

288. Csont T, Viappiani S, Sawicka J, Slee S, Altarejos JY, Batinic-Haberle I, Schulz R: The involvement of superoxide and iNOS-derived NO in cardiac dysfunction induced by pro-inflammatory cytokines. *J Mol Cell Cardiol* 2005, 39:833-840.

289. Zingarelli B, Hasko G, Salzman AL, Szabo C: Effects of a novel guanylyl cyclase inhibitor on the vascular actions of nitric oxide and peroxynitrite in immunostimulated smooth muscle cells and in endotoxic shock. *Crit Care Med* 1999, 27:1701-1707.

290. Nip LH, Uitto VJ, Golub LM: Inhibition of epithelial cell matrix metalloproteinases by tetracyclines. *J Periodontal Res* 1993, 28:379-385.

291. Uitto VJ, Firth JD, Nip L, Golub LM: Doxycycline and chemically modified tetracyclines inhibit gelatinase A (MMP-2) gene expression in human skin keratinocytes. *Ann N Y Acad Sci* 1994, 732:140-151.

292. Cuzzocrea S, Mazzon E, Di Paola R, Esposito E, Macarthur H, Matuschak GM, Salvemini D: A role for nitric oxide-mediated peroxynitrite formation in a model of endotoxin induced shock. *J Pharmacol Exp Ther* 2006, 319: 73-81.

293. Fukuyama N, Takebayashi Y, Hida M, Ishida H, Ichimori K, Nakazawa H: Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. *Free Radic Biol Med* 1997, 22:771-774.

294. Upchurch GR, Jr., Ford JW, Weiss SJ, Knipp BS, Peterson DA, Thompson RW, Eagleton MJ, Broady AJ, Proctor MC, Stanley JC: Nitric oxide inhibition

increases matrix metalloproteinase-9 expression by rat aortic smooth muscle cells in vitro. *J Vasc Surg* 2001, 34:76-83.

295. Eagleton MJ, Peterson DA, Sullivan VV, Roelofs KJ, Ford JA, Stanley JC, Upchurch GR, Jr.: Nitric oxide inhibition increases aortic wall matrix metalloproteinase-9 expression. *J Surg Res* 2002, 104:15-21.

296. Robinson EK, Seaworth CM, Suliburk JW, Adams SD, Kao LS, Mercer DW: Effect of nos inhibition on rat gastric matrix metalloproteinase production during endotoxemia. *Shock* 2006, 25:507-514.

297. Zaragoza C, Soria E, Lopez E, Browning D, Balbin M, Lopez-Otin C, Lamas S: Activation of the mitogen activated protein kinase extracellular signal-regulated kinase 1 and 2 by the nitric oxide-cGMP-cGMP-dependent protein kinase axis regulates the expression of matrix metalloproteinase 13 in vascular endothelial cells. *Mol Pharmacol* 2002, 62:927-935.

298. Zaragoza C, Balbin M, Lopez-Otin C, Lamas S: Nitric oxide regulates matrix metalloprotease-13 expression and activity in endothelium. *Kidney Int* 2002, 61:804-808.

299. Ridnour LA, Windhausen AN, Isenberg JS, Yeung N, Thomas DD, Vitek MP, Roberts DD, Wink DA: Nitric oxide regulates matrix metalloproteinase-9 activity by guanylyl-cyclase-dependent and -independent pathways. *Proc Natl Acad Sci USA* 2007, 104:16898-16903.

300. Dorman G, Kocsis-Szommer K, Spadoni C, Ferdinandy P: MMP Inhibitors in Cardiac Diseases: An Update. *Recent Patents Cardiovasc Drug Discov* 2007, 2:186-194.

301. Stricklin GP, Jeffrey JJ, Roswit WT, Eisen AZ: Human skin fibroblast procollagenase: mechanisms of activation by organomercurials and trypsin. *Biochemistry* 1983, 22:61-68.

302. Hubbard WJ, Choudhry M, Schwacha MG, Kerby JD, Rue LW, III, Bland KI, Chaudry IH: Cecal ligation and puncture. *Shock* 2005, 24 Suppl 1:52-57.

303. Anel R, Kumar A: Human endotoxemia and human sepsis: limits to the model. *Crit Care* 2005, 9:151-152.

304. Buckley JF, Singer M, Clapp LH: Role of KATP channels in sepsis. *Cardiovasc Res* 2006, 72:220-230.

305. Steinberg J, Halter J, Schiller HJ, Dasilva M, Landas S, Gatto LA, Maisi P, Sorsa T, Rajamaki M, Lee HM *et al.*: Metalloproteinase inhibition reduces lung injury and improves survival after cecal ligation and puncture in rats. *J Surg Res* 2003, 111:185-195.

306. Sariahmetoglu M, Crawford BD, Leon H, Sawicka J, Li L, Ballermann BJ, Holmes C, Berthiaume LG, Holt A, Sawicki G *et al.*: Regulation of matrix metalloproteinase-2 (MMP-2) activity by phosphorylation. *FASEB J* 2007, 21:2486-2495.

307. Tesse A, Martinez MC, Meziani F, Hugel B, Panaro MA, Mitolo V, Freyssinet JM, Andriantsitohaina R: Origin and biological significance of shed-membrane microparticles. *Endocr Metab Immune Disord Drug Targets* 2006, 6:287-294.

308. VanWijk MJ, Nieuwland R, Boer K, van der Post JA, VanBavel E, Sturk A: Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction? *Am J Obstet Gynecol* 2002, 187:450-456.

309. VanWijk MJ, Svedas E, Boer K, Nieuwland R, VanBavel E, Kublickiene KR: Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from healthy pregnant women. *Am J Obstet Gynecol* 2002, 187:1686-1693.

310. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS: Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res* 2003, 109:175-180.

311. Freyssinet JM, Dignat-George F: More on: Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2005, 3:613-614.

312. Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS: Endothelium-derived microparticles impair endothelial function in vitro. *Am J Physiol Heart Circ Physiol* 2004, 286:1910-1915.

313. Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biro E, Nieuwland R, Sturk A, Dignat-George F, Sabatier F, Camoin-Jau L *et al.*: Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2004, 2:1842-1843.

314. Castresana J, Saraste M: Does Vav bind to F-actin through a CH domain? *FEBS Lett* 1995, 374:149-151.

315. Stradal T, Kranewitter W, Winder SJ, Gimona M: CH domains revisited. *FEBS Lett* 1998, 431:134-137.

316. Mezgueldi M, Fattoum A, Derancourt J, Kassab R: Mapping of the functional domains in the amino-terminal region of calponin. *J Biol Chem* 1992, 267:15943-15951.

317. Tang DC, Kang HM, Jin JP, Fraser ED, Walsh MP: Structure-function relations of smooth muscle calponin. The critical role of serine 175. *J Biol Chem* 1996, 271:8605-8611.

318. Zacour ME, Teoh H, Halayko AJ, Ward ME: Mechanisms of aortic smooth muscle hyporeactivity after prolonged hypoxia in rats. *J Appl Physiol* 2002, 92:2625-2632.

319. Jaworowski A, Anderson KI, Arner A, Engstrom M, Gimona M, Strasser P, Small JV: Calponin reduces shortening velocity in skinned taenia coli smooth muscle fibres. *FEBS Lett* 1995, 365:167-171.

320. Obara K, Szymanski PT, Tao T, Paul RJ: Effects of calponin on isometric force and shortening velocity in permeabilized taenia coli smooth muscle. *Am J Physiol* 1996, 270:C481-C487.

321. Matthew JD, Khromov AS, McDuffie MJ, Somlyo AV, Somlyo AP, Taniguchi S, Takahashi K: Contractile properties and proteins of smooth muscles of a calponin knockout mouse. *J Physiol* 2000, 529 (Pt 3):811-824.

322. Nigam R, Triggle CR, Jin JP: h1- and h2-calponins are not essential for norepinephrine- or sodium fluoride-induced contraction of rat aortic smooth muscle. *J Muscle Res Cell Motil* 1998, 19:695-703.

323. Parker CA, Takahashi K, Tao T, Morgan KG: Agonist-induced redistribution of calponin in contractile vascular smooth muscle cells. *Am J Physiol* 1994, 267:C1262-C1270.

324. Menice CB, Hulvershorn J, Adam LP, Wang CA, Morgan KG: Calponin and mitogen-activated protein kinase signaling in differentiated vascular smooth muscle. *J Biol Chem* 1997, 272:25157-25161.

325. Je HD, Gangopadhyay SS, Ashworth TD, Morgan KG: Calponin is required for agonist-induced signal transduction--evidence from an antisense approach in ferret smooth muscle. *J Physiol* 2001, 537:567-577.

326. Walsh MP, Carmichael JD, Kargacin GJ: Characterization and confocal imaging of calponin in gastrointestinal smooth muscle. *Am J Physiol* 1993, 265:C1371-C1378.

327. Jiang Z, Grange RW, Walsh MP, Kamm KE: Adenovirus-mediated transfer of the smooth muscle cell calponin gene inhibits proliferation of smooth muscle cells and fibroblasts. *FEBS Lett* 1997, 413:441-445.

328. Lim CC, Zuppinger C, Guo X, Kuster GM, Helmes M, Eppenberger HM, Suter TM, Liao R, Sawyer DB: Anthracyclines induce calpain-dependent titin proteolysis and necrosis in cardiomyocytes. *J Biol Chem* 2004, 279:8290-8299.

329. Kinoh H, Sato H, Tsunezuka Y, Takino T, Kawashima A, Okada Y, Seiki M: MT-MMP, the cell surface activator of proMMP-2 (pro-gelatinase A), is expressed with its substrate in mouse tissue during embryogenesis. *J Cell Sci* 1996, 109 (Pt 5): 953-959.

330. Behrendtsen O, Alexander CM, Werb Z: Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. *Development* 1992, 114:447-456.

331. Baxter BT: Could medical intervention work for aortic aneurysms? *Am J Surg* 2004, 188:628-632.